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Comparison of Compatibility of Streptococcus Starters in Reconstituted Nonfat Dry Milk and a Whey-Based Bacteriophage Inhibitory Medium

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COMPARISON OF COMPATIBILITY OF STREPTOCOCCUS STARTERS IN RECONSTITUTED NONFAT DRY MILK AND A WHEY-BASED BACTERIOPHAGE INHIBITORY MEDIUM

by

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ABSTRACT

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by

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Compatibility of mixed cultures of Streptococcus lactis and Streptococcus cremoris were studied by the phage tracer technique in 10% nonfat dry milk and a whey-based phage inhibitory medium. Methods of membrane dialysis and differential enumeration were tried and abandoned. Dominance occurred in nonfat dry milk and in the whey-based medium during a continuous fermentation period within a pH range 6.8 to 5.2. The dominance pattern of two-strain combinations in 10% nonfat dry milk was different from that in the whey-based media. Dominance varied from one strain to another in no consistent sequence in some combinations. Dominance patterns of combinations were compared with patterns of stimulation and inhibition.

(83 pages)

INTRODUCTION

Streptococcus lactis and Streptococcus cremoris as starter cultures are used in the dairy industry to produce lactic acid in cheese production. Production of sufficient lactic acid prevents undesirable fermentations causing abnormal fruity, rancid, or putrid flavors and gas formation. It also retards staphylococcal growth and toxin formation (Elliker, 1951; Vedamuthu and Reinbold, 1967). Multiple and/or mixed strain cultures are used in the United States to produce desired qualities (taste and texture) and to prevent bacteriophage attack. Bacteriophage or phage activity has been the most important cause of insufficient acid production (Henning et al., 1965; Moseley and Winslow, 1959; Whitehead and Hunter, 1941). The interaction between two different lactic strains may vary from marked stimulation to inhibition. If strain dominance occurs, one single bacteriophage race can cause failure of acid production necessary to produce normal cheese under controlled time conditions. Thus strain compatibility is important, especially in the preparation of frozen concentrated cultures or where newer pH controlled culturing conditions are used.

The objective of this study was to determine any alteration of strain balance of starters grown in a whey-based bacteriophage inhibitory medium as compared to growth in reconstituted nonfat dry milk (NDM).

REVIEW OF LITERATURE

Dominance Among Lactic Streptococci

When two different strains of lactic streptococci are propagated together, one strain population will eventually outnumber the other strain and thus becomes responsible for producing most of the lactic acid in cheese production (Collins, 1961; Czulak and Hammond, 1954; Nichols and Ineson, 1947). Once the microbial population is dominated by one strain, a single bacteriophage race can lyse the bacteria and seriously retard the production of lactic acid. Collins (1952 b) demonstrated that between 75 and 90% of the population must be susceptible to permit bacteriophage to cause appreciable reduction in acid production of a starter culture. Even then, the lag in acid production was only about 1 or 2 h. A sequence of domination occurred in mixed multiple starters (Collins, 1955 b, 1961; Lightbody and Meanwell, 1955). After each event of mass lysis of the dominant strain, the next in order became responsible for acid production. Each culture dominated all the cultures below it in a definite reproducible order. Addition of bacteriophage active against the final fast acid-producing strain retarded acid production. Collins (1955 a) found that phage-inactivated strains usually required an incubation period of 24 h or

more before a secondary growth (phage-resistant cells) resumed active production. Even then the secondary growth consisted of an unstable population of sensitive cells, bacteriophage, and resistant cells that was undependable in acid production.

Dominance occurs as a result of antibiotic production and/or competitive growth (Collins, 1961; Hoyle and Nichols, 1948; Lightbody and Meanwell, 1955).

Dominance by an antibiotic producing culture will generally occur within one 16 h propagation. Compatibility studies suggest variability in the subdominants' sensitivities to the antibiotic (Collins, 1961). Two known antibioties are produced; some strains of Streptococcus lactis produce nisin (Mattick and Hirsch, 1947), and a few strains of Streptococcus cremoris and Streptococcus lactis subsp. diacetilactis produce diplococcin (Oxford, 1944). Hirsch (1952) had concluded that culutres of S. lactis and S. cremoris produced antibiotics directed against the other, but Collins (1961) found no relationship between species and antibiotic sensitivity patterns. The strains of S. lactis did not produce an antibiotic directed primarily against S. cremoris or vice versa. The antibiotic producing strains were resistant only to their own type of antibiotic. The non-antibiotic producing strains were sensitive to either nisin or diplococcin.

In the experiments by Collins (1961) with non-antibiotic producing cultures 23% of 190 combinations exhibited slight dominance in one day, but generally several days or even weeks of daily pro-

pagation were required before marked dominance became evident. After two weeks 80% of the combinations exhibited some dominance. Lightbody and Meanwell (1955) were able to detect shifts of population balance in one culture mixture as early as 4-6 h by the method of phage-flooding of enumeration plates. No heat-stable inhibitory substance could be identified as the cause.

Domination among non-antibiotic producing cultures and among cultures producing the same antibiotic is the result of competitive growth. Collins (1955 b) demonstrated that the tolerance to lactic acid by the different strains and the ability to dominate might be related; that is, lower pH values retarded least the cultures with the greatest powers of domination. The cultures with the least ability to dominate in skim milk were the least active in the production of lactic acid. But by using an enriched skim milk as a growth medium, the direction of domination in some cases was reversed (Collins, 1961). Thus, the results of Collins indicated that small differences in lag phase, growth rate, tolerance to fermentation end-products, and/or nutritional requirements rather than small undetected amounts of antibiotics as suggested by Lightbody and Meanwell (1955) determined which non-antibiotic producing strain would dominate. Hence, Collins reasoned that the competitive ability of lactic streptococci may be influenced by differences in the physiological state of the microorganism at the time of testing and by differences in the composition of the growth medium.

Investigators wanted to know which factors determined dominance. Contrary to the findings of Nichols and Ineson (1947), Collins (1955 b) and Reddy et al. (1971) found no correlation between temperatures of incubation and dominance patterns except that dominance may have occurred more rapidly when the culture was grown at 32 C than at 22 C (Collins, 1961). Wide differences in the initial cell number of the component strains had little or no effect in determining the ultimate population balance (Collins, 1955 b; Hoyle and Nichols, 1948; Reddy et al., 1971). Domination was changed by altering the composition of the growth medium (Collins, 1961; Gilliland, 1972).

Gilliland (1972) reported that a shift of the population ratio may or may not affect the lactic acid production of a mixed cheese starter. A change in phage susceptibility of the starter sometimes occurred without an alteration in lactic acid production. Gilliland (1972) demonstrated that the composition of the growth medium rather than growth with or without automatic pH control was responsible for a population shift.

Czulak and Hammond (1954) believed that combinations of S. lactis and S. cremoris were more compatible than two strains of S. cremoris, however Lightbody and Meanwell (1955) and Collins (1961) found no relationship between species and the ability to dominate.

With proper selection of strains, mixtures of lactic streptococci have been subcultured compatibly for periods up to 2 weeks (Collins, 1961) and to 4 weeks (Czulak and Hammond, 1954). But

results prove that it is uncommon for strains of rapid acid-producing lactic streptococci to grow harmoniously for long periods in a fixed proportion (Collins, 1961; Czulak and Hammond, 1954; Lightbody and Meanwell, 1955).

Techniques Used To Identify Dominance

The phage tracer technique has been used by Collins (1961), Czulak and Hammond (1954), Gilliland (1972), Henning et al. (1964), Lightbody and Meanwell (1955), Nichols and Ineson (1947), and Vedamuthu et al. (1966) in their experiments to identify component strain populations in starter mixtures. The procedure of Collins (1961) is described in Methods and Procedure.

A differential enumeration technique has been developed by Reddy et al. (1969, 1972). By using a differential agar medium with a diffusible (K_2HPO_4) and a non-diffusible ($CaCO_3$) buffer system, milk as the sole carbohydrate source, and arginine as the specific substrate, S. lactis were separated from S. cremoris. Colonies of S. cremoris appeared yellow with a yellow zone around them due to acid produced from the fermentation of lactose in the milk; but S. lactis also hydrolyzed the arginine with the liberation of NH_3 which reversed the pH indicator color to purple. Therefore, S. lactis appeared as white colonies on the purple medium.

Associative Growth

The term associative growth relation has been used to describe the shift in population balance of a multiple and/or mixed starter due to domination (Reddy et al., 1972). The term has also been used to describe symbiotic and antagonistic growth patterns as measured by acid production (Dahiya and Speck, 1963; Kothari and Nambudripad, 1973; Kothari et al., 1971). Experiments by Gilliland (1972) suggested that dominance and the symbiotic and antagonistic growth patterns are separate phenomena. Alterations in strain balance resulted in a symbiotic interaction, an antagonistic interaction, or no change in acid production. Thus, phage inter-relationships, dominance, symbiotic and antagonistic interactions as well as proteolytic and acid production activities should be considered in selection of strains for a multiple and/or mixed starter.

The milk filtrate from one lactic culture can often stimulate the acid production of another culture. Enzymatic digests of proteins and extracts of certain plant and animal tissues have also been used to accelerate acid production of lactic streptococci (Anderson and Elliker, 1953; Garvie and Mabbit, 1956). Generally, the supplements stimulated the slow much more than the fast acid producers (Dahiya and Speck, 1962). Dahiya and Speck (1963) experimented with the filtrate of a slow acid-producing strain which stimulated a fast acid producer. The major stimulant was identified as adenine.

Dahiya and Speck (1964) concluded that milk is deficient in nucleic acid derivatives for optimum growth of lactic streptococci.

Kothari et al. (1971) found that the acid production of active paired cultures was at least 4% higher at all time intervals than the acid production of either culture grown alone, but the maximum recordable stimulation occurred at about 8 h at 30 C. In all cases the cell-free filtrate of one culture stimulated the acid production of the companion culture, but the reverse combinations showed no stimulation. The stimulatory action was one sided and not necessary for survival of either culture; hence, no true symbiosis existed. Increasing the amount of filtrate progressively increased acid production. Maximum stimulation was approached when 13% filtrate was added; however, as little as 0.7% filtrate effected higher acid production. In further investigations Kothari and Nambudripad (1973), using cultures KH, HC, and R_1, concluded that (1) casein was necessary for production of the stimulatory material, (2) casein was essential for proper utilization of the stimulatory material, (3) 2.0-2.5% casein was necessary for associative growth to occur, and (4) the stimulatory material was guanine or a guanine-like substance.

Interbiosis Studies Using Dialysis Culture

Dialysis culture can be used to study associative growth rela-

tionships and dominance. Two or more populations of organisms are placed on opposite sides of one or more membranes so that their diffusible metabolites can interchange.

Dialysis culture is a very complicated subject. Schultz and Gerhardt (1969) have written an outstanding review of the principles. A summary of some important factors to interbiosis in a liquid-liquid system follows.

The pores of membranes are usually filled with a liquid. Solute molecules either diffuse through the passageways or are carried through by a flow of liquid. Although electrostatic charge and other factors may have an influence upon diffusion through the membrane, it is mainly the largest passageway in the membrane filter which determines the maximum size of a molecule which can penetrate.

The ability of a membrane to filter bacteria is dependent on a pore structure smaller in size than the organisms and the absence of extreme differential pressure. There is evidence that bacteria can penetrate, possibly grow through, a filter membrane with pores far smaller than the size of the cells. Therefore, the duration of incubation time may be important.

Fick's law of diffusion has been adapted to membrane permeability.

$$N = \frac{D^{\cdot} Ap}{Lp} \Delta S$$

N = rate of permeation (g/h)

$D^{\cdot}$ = diffusivity of the solute in the liquid (cm^2/h)

Ap = pore area of the total membrane area available for diffusion (cm^2)

Lp = length of the diffusion path in the pores (cm)

ΔS = concentration differences across the film (g/cm^3)

The diffusivity within the membrane, the pore areas, and the lengths of the diffusion pathways are unmeasureable. Therefore these unknown factors are combined to give an empirical factor, the permeability coefficient (Pm) which can be experimentally determined for each membrane and solute.

$$N = Pm\ Am\ \Delta S$$

Pm = permeability coefficient (cm/h)

Am = total area of the membrane (cm^2)

Thus, the rate of molecular diffusion through an inert porous membrane is directly proportional to the permeability coefficient, membrane area, and the concentration difference across the membrane.

As the prime purpose of the membrane is to retain the microbial cells in the fermentor chambers, the average pore size should be 15 times the diameter of the largest diffusible metabolite. Durability is probably more important than the pore size.

The rate of dialysis is strongly influenced by liquid turbulence at the membrane surface. The Pm can be increased as much as two or three times by vigorous stirring or high flow rates near the membrane.

METHODS AND PROCEDURES

Cultures

Typical culture strains used in the cheese industry were obtained from Dairy Products Laboratory (1750 Folsom St., San Francisco, CA 94103) as lyophilized powders. The strains were designated as Streptococcus lactis #1 and #2 and Streptococcus cremoris #3, #4, #5. A second set of S. cremoris, single-strain cultures #1, #2, #3, KH, and R_1, and filtrates containing homologous phage were obtained at a later date. Three cultures, AM_2, ML_8, and S. lactis C_2 were obtained from the stock cultures of Utah State University. Hereafter, the cultures shall be identified as Sl 1, Sl 2, Sc 3, Sc 4, Sc 5, #6, #7, #8, KH, R, AM_2, ML_8, and C_2.

Cultures were propagated in sterile 11% nonfat dry milk (NDM). After 8 h of incubation at 30 C, the cultures were refrigerated until transferred. Transfers were made weekly.

Due to loss of activity after four to six transfers, the procedure was changed. Cultures were grown for 8 to 12 h at 30 C and 2% inoculum was transferred to fresh medium. The new series was refrigerated and incubated just before transfer. Transfers were made twice weekly. If the cultures lost their activity, new cultures were propagated from the powder.

Two daily transfers were made preceding each experiment.

Phage Filtrates

Phage filtrates were prepared as outlined by Cheng:

> Fifty milliliters of sterilized and cooled Elliker lactic broth supplemented with one percent sterile 1 M $CaCl_2$ was inoculated with 1 percent culture and incubated at 30 C for 2 to 3 hr until broth was slightly turbid indicating an early log phase of culture development. The cultured broth was infected by one percent of homologous phage filtrate. The phage infected broth was incubated overnight at room temperature (25 C). In most cases, the lysates were clear when compared to a non-infected control culture. The phage lysates were centrifuged for 15 minutes and filter-sterilized sequentially through two membrane filters with average pore diameter of 45 and 22 um (Millipore Filter Corp., Bedford, Mass.). This treatment removed bacterial cells and other particles. The resulting phage filtrates were maintained at 2 to 4 C until used.
>
> The phage titer was estimated by a dilution end-point method in sterile litmus milk. (Cheng, 1970, p 16-17)

Preparation of the Whey-Based Bacteriophage Inhibitory Medium

The medium was made according to the formula given by Ausavanodom (1974), Table 1. The reconstituted medium was sterilized at 121 C for 15 min. The medium was stirred during dispensing to insure equal distribution of precipitated salts and lactoalbumins.

Table 1. Ingredients of the whey-based phage inhibitory medium

Ingredients	1000 ml
NaH_2PO_4	10.26 g
Na_2HPO_4	9.75 g
Ardamine "YES" [a]	2.00 g
NZ Amine NAK [b]	1.00 g
$MgSO_4$	0.05 g
$FeSO_4$	0.01 g
$MnSO_4$	0.01 g
NaCl	0.01 g
Whey (dehydrated) [c]	72.00 g
Water	904.90 ml

[a]Ardamine "YES" or Autolyzed yeast extract powder by Yeast Products Inc., 455 Fifth Avenue, Paterson, N.J.

[b]Type NAK, Lot No. 90K11, Sheffield Chemical A Division of Kraftco, R.D. 3, Oneonta, N.Y.

[c]Hi-Land whey powder, spray process; Extra grade edible. Hi-Land Dairy, 700 Vine St., Murray, Utah

Preparation of NDM

Ten or eleven percent NDM was autoclaved for 15 min at 116 C.

Experiments in Membrane Dialysis

The use of membrane dialysis or diffusion chambers was analyzed as a means to study compatibility of cheese starters. Two cylindrical fermentors (250 ml capacity for each) with side arms were separated by a filter membrane with an average pore size of 1.2 or 0.45 um (Millipore HAWP made by Millipore Filter Corp., Bedford, Mass.). The dialysis chambers were filled with distilled water and sterilized. The rate of diffusion of lactic acid in water and in NDM was measured by adding 2 ml of lactic acid to the fermentor and recording the pH changes in the opposing diffusion chamber, the reservoir. In the next experiments 1% starter was added to the fermentor. A membrane with a 0.45 um pore size separated the dialysis chambers. Both chambers were agitated gently with magnetic stirrers and incubated at 22 C for 24 h. A second set of chambers using a membrane with a 1.2 um pore size and without agitation was incubated for 24 h at 30 C. The pH changes of the reservoir were monitored and recorded.

Technique Using Differential Enumeration

The methods as described by Reddy et al. (1969, 1971, 1972) were followed except Dupont Carboxy Methol Cellulose (CMC)

grade P-75 lot XH was used because of the unavailability of Carboxy Methyl Cellulose (CMC) Cekol MV, Uddeholm, Sweden, or of Dupont CMC grade P-754. The differential agar medium for separating S. lactis and S. cremoris was most commonly used. In a beaker, 15 grams of agar was suspended in 500 ml of distilled water. In another beaker, 6 grams of Dupont CMC was added to 500 ml of hot distilled water. The two beakers were placed in the autoclave and steam heated at 121 C for 10 min. If the CMC was not completely dissolved, the solution was mixed in a Waring Blender. The two portions were mixed together, and other ingredients were added: 0.5% tryptone, 0.5% yeast extract, 0.4% L-arginine hydrocloride, 0.1% K_2HPO_4, and 0.3% $CaCO_3$. The mixture was steam treated in the autoclave for 10 min. At such time the pH was 6.8 $\pm$0.1. The medium was continuously agitated while being poured into containers in 100 ml quantities. It was then sterilized at 121 C for 15 min. When the medium was needed, it was remelted and tempered to 55 C in a water bath. Five milliliters of a sterile 11% NDM and 2.0 ml of a 0.1% bromocresol purple (BCP)-water solution were added to every 100 ml of medium. The contents of the bottle were mixed thoroughly but without incorporation of air in order to ensure homogeneous suspension of the insoluable $CaCO_3$. The plates were poured and allowed to solidify. Spread plates were made of test cultures and incubated in a candle oats jar at 32 C

for 36-48 h (Reddy et al., 1969). The purpose of the burning candle and the moist oats was to reduce the oxygen level and maintain a CO_2 tension.

Determining Antibiotic Production

Each culture was tested for the production of antibiotics in a manner similar to that of Collins (1961). Agar plates of Tomato Juice Agar and lactic agar (Elliker Broth with 15% agar added) were prepared by pouring the media and allowing it to solidify. The plates, opened and inverted, were dried in the 30 C incubator for about 8 h. A lawn of each culture was prepared on each medium by spreading one or two drops of the culture over the agar surface with a glass "hockey stick." One drop of each culture to be tested for antibiotic production was placed on the culture lawn. Any zone of inhibition after 24 or 48 h of incubation at 30 C was interpreted as antibiotic production.

Also, the production of antibiotics would be detected by a drastic inhibition of acid production with the use of a particular culture filtrate in the associative growth experiments.

Associative Growth as Measured by Acid Production

Screening of culture combinations for associative growth

Cultures were grown singly and in pairs in NDM. The total inoculum was 1% (½% each of the pair). The pH of all cultures was checked at 6, 10, and 12 h of incubation at room temperature (22-25 C). The culture tubes were placed in an ice water bath to stop acid production during the time of inoculation and pH measurements. The temperature of the cultures was raised to 25 C by running warm water in the water bath and maintained at room temperature during incubation periods. The pH electrode was washed with distilled water between every measurement to minimize contamination.

Changes in procedures afterwards were as follows. The best time to measure differences varied with the initial physiological state and activity of the cultures, therefore culture tubes containing BCP (range 5.2-6.8, yellow-purple color reaction) were used as reference of acid development. Once the definite yellow appeared in any of the tubes, the pH of all tubes was read.

A similar experiment using the whey-based medium was conducted. Reference tubes with BCP were used.

If the pH value of the pair deviated much above or below the pH value of the fastest acid producer (the single culture),

the results were compared with proceeding experiments for possible associative growth patterns.

Experiments using pasteurized cultures to stimulate acid production

The first few experiments employed the use of a bacteria-free filtrate. The filtrate was prepared by incubating pure cultures at 30 C for 24 h; the cultures were placed in a centrifuge for 30 min at a relative adjuctment to pH 7.0 with NH_4OH was Seitz filtered.

Dahiya and Speck (1962) found the stimulatory material to be heat stable. Kothari, et al. (1971) heat treated the cultures at 45-50 C for 30 min. Therefore it was decided to pasteurize the cultures and use them without filtration. This method eliminated clogged Seitz filters and shortened preparation time.

The cultures were grown in 11% NDM for 24 h at 30 C. The cultures were then neutralized with NH_4OH and pasteurized in a water bath at 62 C for 30 min. One milliliter of the appropriate pasteurized culture was added to 8 ml of sterile 11% NDM. The tubes were inoculated with 1 ml of a 1:10 milk dilution of fresh active single cultures or 0.5 ml of each culture when grown in pairs. The inoculated tubes were incubated in a water bath at 30 C for 8 h or until BCP reference tubes indicated sufficient acid development (method used is noted in

test results). At which time, the titratable acidity as well as the pH was determined. The average culture produces enough acid to change the pH of milk from 6.8 to 4.9, a 1.9 unit decrease; therefore, any deviation greater than 10% or 0.20 in pH readings from the control was considered as possible associative growth. The extent of associative growth as measured by the titratable acidity was determined by the following formula (Kothari et al., 1971):

$$\text{Percent associative growth} = \frac{A - B}{B} \times 100$$

Where A = ml N/10 NaOH required to neutralize the stimulated or inhibited cultures. B = ml N/10 NaOH required to neutralize the fastest acid producing control.

Any deviation of 10% or more was considered as an associative growth pattern.

The methods of the last experiment were changed as follows: The control consisted of sterile NDM which was pasteurized. A second control of NDM acidified with lactic acid, neutralized to pH 7.0, and pasteurized, was used as in preceding experiments. Two milliliters of the controls or the pasteurized cultures were added to tubes of 8 ml of sterile 11% NDM in respective order in an effort to obtain maximum stimulation. Each series of tubes was then inoculated with 1 ml of a 1:10 milk dilution of the active culture. After 8 h of incubation at 30 C, the

titratable acidity was read and the percent associative growth was calculated.

Tests Involving Prolonged Fermentation at a Relatively Constant pH

Ten milliliters of skim milk with 0.015% BCP was innoculated with 1% of a paired culture (0.5% of each) or 1% of a single culture (the control). The milk tubes were incubated at 30 C. As the color indicator changed, the tubes were neutralized with 20% NH_4OH. The NH_4OH was dispensed drop by drop from a 50 cc syringe pump (flow rate = 0.7 ml/min). After 18 h of incubation neutralization was terminated, and results were charted. Any stimulation by mixtures was noted by an increase in the rate of acid production (determined by the slope) and by an increase in the total acid production over the controls.

A second experiment used pasteurized cultures to stimulate acid production of active single cultures. The cultures were grown in 11% NDM with .015% BCP for 24 h at 30 C. The cultures were neutralized to pH 7.0 with 28-30% NH_4OH and pasteurized in a water bath at 62 C for 30 min. One control was prepared by adding lactic acid to sterile 11% NDM until pH 4.7 was reached; it was then neutralized and pasteurized in the normal manner. A second control was sterile 11% NDM which was pasteurized. Two

milliliters of the appropriate pasteurized culture or control was added to 7 ml of sterile 11% NDM with BCP. Each set of tubes was inoculated with 1 ml of the inoculum (1% total of single cultures). The milk tubes were incubated at 22 C in an effort to lengthen the time intervals between neutralization and maintain a more constant pH range. The tubes were neutralized as before. The acid production rate of the single strains in the presence of the 24 h, neutralized, pasteurized culture was compared to the acid production rates of the two controls for a 32 h period.

The last procedure was repeated using the whey-based medium. A broken needle on the syringe pump was replaced with a 22 G $10\frac{1}{2}$ inch syringe needle, bent to a 90° angle without restricting flow. The flow rate was 0.7 ml/min and 100 drops/ml. No attempt was made to compare acid-production activity between tests; only the patterns of stimulation or inhibition were compared.

Experiments Using the Phage Tracer Technique

The procedure of Collins (1961) was as follows: The culture mixture was allowed to grow in the test medium according to set conditions (temperature, incubation time, number of propagations, etc.). A sample to be tested for dominance was drawn and inoculated into four containers of

sterilized Litmus Milk. One container served as a control. The second, containing strain-specific phage for each strain in the mixture, measured the bacteriophage sensitivities of the cultures. Containers three and four, to which was added one strain-specific phage in respective order, identified the dominant strain through subsequent failure in acid production. Collins (1961) labelled three gradations of retardation: (1) reduced and weakly coagulated, (2) reduced and not coagulated, and (3) neither reduced nor coagulated after 7 h at 32 C to indicate very slight, slight, and marked dominance respectively.

Gilliland (1972) using a similar method measured dominance as the time-delay required by the culture to reach pH 5.0.

In my experiments, cultures #6, #7, #8, KH, and R plus their homologous phage were used. The phage were strain-specific races except #7 which exhibited the nascent phenomenon by attacking KH when grown with culture #7. The nascent phenomenon is a condition where the bacteriophage absorb to the bacterium and prevent bacterial multiplication of a culture but are unable to assume command of phage synthesis or phage assembly within the bacterial cell (Collins, 1952 a).

Culture mixtures were propagated in 10% NDM and the whey-based medium. The mixtures were prepared by inoculating the test media with 2% of each respective culture; these were incubated at room temperature for 8 h. The mixtures were trans-

ferred every 8 h thereafter. Every 24 h samples were drawn and inoculated into four tubes according to the method of Collins (1961) except NDM was used and the pH of each tube was measured by the failure of the pH to drop in either tube 3 or 4. In this manner an active culture was maintained in a pH range 6.8 to 5.2, the visual endpoint range of BCP.

RESULTS

Results of Membrane Dialysis

When 2 ml of lactic acid was added to one side of the diffusion chambers filled with distilled water and separated by a 0.45 um membrane, a rapid decrease in pH in the opposite chamber occurred within 10 min. The rate of diffusion was greatly reduced when tap water was used (Table 2). When 10% NDM solution was used in the chambers, no pH change across the membrane occurred in 24 h.

Table 2. Rates of lactic acid diffusion across a membrane filter (pore size 0.45 um) between two dialysis chambers as measured by differences in pH readings of one chamber taken in 10 min intervals

	Distilled Water			Tap Water	
Trials:	1	2	3	1	2
Time (min)	Decrease in pH units/10 min				
10	1.60	1.80	1.65	.55	.55
20	.20	.12	.15	.42	.48
30	.10	.11	.18	.24	.27
40	.05	.03	.05	.19	.20
50	---	.04	.04	.17	---
60	---	.01	.03	---	---
70	---	---	.05	---	---

Sterile NDM on one side of the membrane was inoculated with cultures #7, #8, and R, then incubated with agitation at 22 C. The milk in the inoculated fermentor coagulated; the pH was 4.7. The opposite chamber, the reservoir, was still the original pH 6.6 after 24 h.

No exchange dialysis of lactic acid in 24 h occurred even when a membrane with a 1.2 um pore size was utilized. Both chambers coagulated in another 8 h once the fermentor and reservoir chambers were agitated. Due to the slow pH change across such membrane systems using NDM substrate, no further experiments were conducted.

The failure of lactic acid to enter into exchange dialysis may be due to simple physical clogging of the membrane filter. At pH 6.6 lactic acid is almost completely dissociated to lactate and hydrogen ions. At pH 4.8, 90% of the lactic acid is still ionized releasing hydrogen ions. However, due to the total lack of any pH change, the hydrogen ions may be absorbed or even chemically combined with the milk components in some manner. If the hydrogen ions existed in a free dissociated state, they would likely penetrate the membrane.

Schultz and Gerhardt (1969) calculated that the cell density of a dialysis culture can be increased many times that of a nondialysis culture with proper conditions. Dialysis culture possibly can be used for preparation of concentrated starters

and further studies of dominance upon selection of proper membrane material and culture conditions.

Results of Differential Enumeration

The use of the differential media was discarded when difficulties were encountered. In the broth, both growth and differentiation of pure cultures were excellent. However on the solid media growth or differentiation was not constant. The concentration and even elimination of CMC had no noticeable effect on results. The degree of differentiation was altered by changing the concentrations of the milk and/or K_2HPO_4. The sugar-arginine-buffer balance necessary in the medium to obtain the proper color changes of the indicator seemed to be a critical factor for differentiation of two strains. Properly suspending the $CaCO_3$ without incorporating air into the medium was also a problem. The degree of inhibition of growth by oxygen was not known or measured. In one experiment a L-leucine and D-leucine mixture was used because of immediate demand. Afterwards it was learned that D-leucine, an auto-inhibitor of lactic streptococci, was the most probable reason for that failure (Gilliland and Speck, 1968). During another trial experiment, the temperature at which the milk was added to the medium was believed to be a critical factor. Poor growth and

inconsistent differentiation was observed. The exact reason for other failures was not determined. Possibly the use of the specified brand of CMC is important in obtaining results.

Antibiotic Production

None of the cultures used produced antibiotics. The results were confirmed by the lack of inhibition of acid production by the pasteurized cultures in the associative growth experiments.

Screening the Culture Combinations For Associative Growth

The two sets of lactic streptococci strains from DPL plus C_2 and ML_8 were used in the first experiment. S1 2 was eliminated due to poor acid development. Three controls of each single culture and two tubes of all possible combinations were stimultaneously propagated in milk. Differences of pH readings from that of the controls was used to measure associative growth. The optimum incubation time at which significant pH differences occurred needed to be determined. Measurements at 6 and 12 h produced insignificant differences. At 10 h, pH differences of at least ±0.2 existed. Combinations #6 + C_2, R + C_2, #7 + #8,

#7 + R, and #7 + C_2 had a greater pH drop than the controls indicating a stimulatory relationship. Combinations Sc 3 + R, #6 + ML_g, and Sc 5 + R seemed to retard acid production of the most active strain as in an antagonistic relationship (Table 3).

Efforts to repeat the associative growth patterns failed. The combinations were no more active than the most active single culture. In fact, combination #7 + #8 had an average pH 5.3 while #7 averaged pH 4.75. Thus #7 seemed to be retarded by #8 which had a pH 5.6.

In a third experiment reference tubes with BCP were used because the rate of acid development varied according to the initial physiological state of the innoculum. No pH differences greater than ±0.1 were observed between controls and combinations of the 13 strains. They were considered insignificant (Table 4).

The latter experiment was repeated using the whey-based medium. No stimulation occurred; but often if a single strain had not yet reached its full capacity of acid production at the time of testing, the combination with a very active strain tended to decrease the total acid production. An example is Sl 1 + #7 (Table 5). This trend, although not as noticeable, did exist when milk was used.

Table 3. Possible associative growth patterns in nonfat dry milk determined by comparing pH readings of the two single cultures and the combination of the active pair. Cultures incubated incubated at 25 C for 10 h in NDM. Any deviation ±0.2 in pH readings from the fastest single acid producer was considered as possible stimulation or inhibition. Three controls of each single culture and two tubes of all possible combinations were simultaneously propagated in 10% NDM substrate.

Active Cultures

Active Cultures		Active Cultures											
		C^a	Sl 1	Sc 3	Sc 4	Sc 5	#6	#7	#8	R	KH	C_2	ML_8
	C^a	6.5	5.1	5.2	5.1	5.2	5.7	5.6	5.5	5.7	5.3	5.9	6.4
			5.0	5.2	4.8	5.1	5.8	5.3	5.3	5.6	4.9	6.1	6.5
			5.4	4.9	5.0	5.0	5.8	5.5	5.3	5.9	5.2	6.0	6.5
	Sl 1	5.1		5.2	4.9	5.4	5.3	5.3	4.9	5.4	5.1	5.4	5.2
		5.0		4.9	5.0	4.8	4.9	5.3	5.3	5.4	5.0	5.4	5.4
		5.4											
	Sc 3	5.2			4.7	4.9	5.1	5.2	5.0	5.3	5.0	4.9	5.3
		5.2			4.8	4.9	4.9	5.1	5.1	5.4	4.9	5.0	5.2
		4.9											
	Sc 4	5.1				4.8	4.9	4.8	4.9	4.7	4.8	4.9	4.9
		4.8				4.8	4.9	4.9	4.7	4.8	4.8	5.0	5.4
		5.0											
	Sc 5	5.2					5.2	5.2	5.0	5.9	5.0	5.3	5.3
		5.1					5.3	5.4	5.1	5.5	5.2	5.2	5.3
		5.0											
	#6	5.7						5.4	5.6	5.6	5.3	5.6	6.1
		5.8						5.5	5.2	5.6	5.4	5.4	6.0
		5.8											
	#7	5.6							5.0	5.4	5.0	5.1	5.7
		5.8							4.9	4.9	4.9	5.2	5.4
		5.5											
	#8	5.5								5.5	5.2	5.4	5.5
		5.3								5.5	5.0	5.0	5.5
		5.3											
	R	5.7									5.6	5.3	5.6
		5.6									5.1	5.3	5.2
		5.9											
	KH	5.3										5.1	5.2
		4.9										5.2	5.3
		5.2											
	ML_8	6.4											6.0
		6.5											6.0
		6.5											

[a]Control

Table 4. Possible associative growth patterns in nonfat dry milk determined by comparing pH readings of two single cultures and combination of pair. Cultures incubated at 30 C for 6.5 h. Any differences of pH between the fastest single acid producer and the combination greater than ±0.2 was considered as possible associative growth. Example: pH values of #8 + Sc 3 were compared with pH values of control combinations #8 + #8 and Sc 3 + Sc 3

Active Cultures

	Sl 1	Sl 2	Sc 3	Sc 4	Sc 5	#6	#7	#8	KH	R	AM_2	C_2	ML_8
Sl 1	4.80	4.85	4.85	4.80	4.85	4.75	4.75	4.65	4.80	4.80	4.70	4.85	4.65
	4.90	4.75	4.85	4.80	4.85	4.70	4.75	4.70	4.85	4.80	4.75	4.80	4.75
Sl 2		4.90	4.80	4.85	4.90	4.75	4.75	4.70	4.80	4.90	4.70	4.90	4.75
		5.10	4.80	4.85	4.90	4.80	4.80	4.80	4.85	5.00	4.75	5.00	4.75
Sc 3			4.95	4.95	4.90	4.80	4.90	4.90	4.85	4.85	4.75	5.00	4.80
			5.10	4.85	4.95	4.70	4.90	4.80	4.95	4.80	4.80	5.00	4.70
Sc 4				4.90	4.90	4.75	4.80	4.85	4.80	4.80	4.70	4.85	4.80
				5.00	4.90	4.75	4.80	4.80	4.85	4.80	4.75	4.90	4.75
Sc 5					5.00	4.75	4.75	4.75	4.85	4.90	4.70	4.95	4.70
					5.30	4.75	4.85	4.80	5.00	4.90	4.70	4.90	4.70
Active Cultures #6						4.65	4.70	4.70	4.75	4.70	4.65	4.70	4.75
						4.85	4.70	4.65	4.75	4.75	4.65	4.80	4.70
#7							4.70	4.70	4.90	4.80	4.75	4.90	4.70
							5.00	4.75	4.80	4.80	4.70	4.80	4.60
#8								4.70	4.70	4.80	4.70	4.85	4.70
								4.95	4.70	4.80	4.70	4.80	4.65
KH									4.80	4.85	4.65	4.80	4.70
									4.90	4.80	4.70	4.85	4.75
R										4.85	4.80	4.85	4.70
										5.00	4.70	4.80	4.70
AM_2											4.65	4.75	4.75
											4.70	4.70	4.70
C_2												5.10	4.75
												5.15	4.80
ML_8													4.70
													4.75

Second decimal place estimated to nearest 0.05

Table 5. Possible associative growth patterns in the whey-based media determined by comparing pH readings of two single cultures and combination of the pair. Cultures incubated at 30 C for 6 h. Any difference of of pH between the fastest single acid producer and the combination greater than ±0.2 was considered as possible associative growth. Example: pH values of #8 + Sc 3 were compared with pH values of control combinations #8 + #8 and Sc 3 + Sc 3.

Active Cultures

Active Cultures	Sl 1	Sl 2	Sc 3	Sc 4	Sc 5	#6	#7	#8	KH	R	AM_2	C_2	ML_8
Sl 1	4.75	5.20	4.90	4.80	4.90	4.95	5.30	5.10	5.10	4.80	4.60	4.70	4.60
		4.80	4.80	4.70	4.80	4.70	5.10	5.15	5.20	4.90	4.65	4.80	4.70
Sl 2		6.10	6.15	5.80	5.50	5.35	6.10	5.90	6.10	5.00	4.80	4.90	4.80
			6.20	4.75	5.60	5.40	6.00	5.90	6.10	5.10	4.70	4.90	4.70
Sc 3			6.10	4.90	----	5.20	6.00	5.10	6.20	4.90	4.65	5.00	4.65
				4.90	5.50	5.35	5.90	5.90	6.10	5.20	5.80	4.90	4.70
Sc 4				4.70	----	4.80	4.80	4.90	4.85	4.80	4.60	4.80	4.65
					4.85	4.70	4.80	4.80	4.90	4.75	4.70	4.70	4.60
Sc 5					----	5.00	5.60	5.40	5.80	4.90	4.70	4.90	4.65
						----	5.35	5.40	5.60	4.90	4.70	4.85	4.60
#6						4.85	5.15	5.30	5.30	4.80	4.70	4.85	4.65
							5.15	5.00	5.20	4.80	4.65	4.80	4.65
#7							6.00	5.80	6.00	5.05	4.65	4.90	4.65
								5.70	5.75	4.90	4.70	4.90	4.65
#8								5.75	5.95	5.00	4.70	4.90	4.65
									5.90	5.00	4.65	4.90	4.65
KH									6.10	5.10	4.70	4.90	4.65
										4.95	4.70	4.90	4.60
R										4.85	4.70	4.85	4.65
											4.60	4.80	4.60
AM_2											4.60	4.60	4.65
												4.65	4.60
C_2												4.80	4.65
													4.65
ML_8													4.60

Second decimal place estimated to nearest 0.05

The Effect of Pasteurized Cultures On Acid Production

In the first experiment, two bacteria-free filtrates were added to NDM in separate tubes. The control culture #8 did not drop below pH 5.9. But #8 was stimulated to acceptable levels of acid production by 1% filtrate from Sc #3 and R.

In the next experiment, the two sets of cultures and AM_2 were used. Culture #7 was excluded because of activity failure. The cultures with filtrates had been incubated $6\frac{1}{2}$ h when two of the BCP reference tubes turned definite yellow, and pH measurements were taken. One percent filtrates from #7 and #8 may have stimulated Sc 5. The pH tests were approximately 0.2 below the control pHs, but the percent associative growth calculated from the titratible acidity (TA) was 13.8% and 11.9%.

In Kothari et al. (1971), the TA continued to increase up to 72 h of incubation, and the maximum percent associative growth occurred in 8 h. Yet in the experiments observed, the maximum pH drop occurred within 8-16 h at 30 C. Therefore, in another experiment, the pH and TA were taken at 8 h. Pasteurized cultures KH, R, and AM_2 drastically inhibited Sl 1. Pasteurized Sc 4 and #7 stimulated Sl 2. Pasteurized Sc 4 inhibited Sc 4. Pasteurized #7 inhibited Sc 5 (Table 6).

It was found in a subsequent experiment (Table 7) that

Table 6. Detection of antagonism or stimulation of single cultures in nonfat dry milk by 10% of a 24 h neutralized, pasteurized culture. Differences of greater than ±0.2 between pH readings or 10% between titratable acidities of controls and cultures plus pasteurized cultures was considered as possible associative growth. BCP reference tubes were yellow after 6.5 h incubation at 30 C

			Pasteurized Filtrates									
		C[a]	Sl 1	Sl 2	Sc 3	Sc 4	Sc 5	#7	#8	KH	R	AM_2
Sl 1	pH	5.0[b]	5.0	5.0	5.1	5.0	5.0	4.95	5.0	6.6	6.6	6.6
	%	(.76)[c]	6.8	6.5	3.7	2.6	1.3	0.	2.6	-72	-73	-72
Sl 2	pH	5.45	5.3	5.5	5.3	5.2	5.2	5.2	5.5	5.3	5.4	5.3
	%	(.60)	1.6	-7.2	1.6	25.8	6.1	17	25	---	15	28
Sc 3	pH	4.8	4.8	4.8	5.1	4.8	4.8	4.8	4.7	4.8	4.7	4.7
	%	(.73)	5.5	1.2	-1.8	4.1	4.1	0.7	6.8	---	2.7	12.3
Sc 4	pH	4.85	4.7	4.8	4.8	5.1	4.8	4.8	4.8	4.8	4.8	4.8
	%	(.76)	5.1	1.2	2.5	-20	3.8	5.1	1.8	6.4	9.1	10.4
Sc 5	pH	4.8	4.7	4.8	4.8	4.8	4.7	5.1	4.6	---	4.7	4.8
	%	(.80)	8.7	4.3	6.3	6.3	6.3	24	6.2	---	5.2	---
#7	pH	4.6	4.6	4.6	4.6	4.7	4.6	4.6	4.6	4.7	4.6	4.6
	%	(.89)	8.5	11.9	-.7	-.7	1.8	2.9	1.8	2.9	2.9	0
#8	pH	4.9	4.8	4.8								
	%	(.77)	9.4	4.9								

[a]Control

[b]The average pH of two controls. Note: other pH readings made to nearest 0.1

[c]The average titratable acidity of two controls

Table 7. The effect of 18% of a 24 h neutralized, pasteurized culture upon single, active cultures in nonfat dry milk measured in terms of percent stimulation or inhibition. Associative growth determined after 8 h incubation at 30 C

	Active Cultures											
	Sl 1		Sc 3		Sc 4		Sc 5		#7		#8	
PC[a]	TA[b]	%	TA	%	TA	%	TA	%	TA	%	TA	%
Milk	---	-	.69	C[c]	.73	C	.46	C	.66	C	.74	C
ANC[d]	.70	C	.68	-1	.72	-1	.45	-2	.66	0	.73	-1
Sl 1	.72	3	.73	6	.75	3	.72	57	.74	12	.74	0
Sl 2	.70	0	.71	3	.73	0	.64	39	.72	9	.73	-1
Sc 3	.67	-4	.72	4	.74	1	.65	41	.73	11	.77	4
Sc 4	.71	1	.72	4	.72	-1	.66	43	.74	12	.73	-1
Sc 5	.70	0	.71	3	.77	5	.64	39	.73	11	.73	-1
#7	.73	4	.72	4	.75	3	.64	39	.75	14	.73	-1
#8	.67	4	.70	1	.73	0	.59	28	.73	11	.71	4
KH	.70	0	.72	4	.74	1	.69	50	.73	11	.73	-1
R	.69	1	.70	1	.73	0	.64	39	.72	9	.72	-3
AM_2	.70	0	.72	4	.70	-4	.65	41	.72	9	.73	-1

[a]Pasteurized Cultures
[b]Titratable Acidity
[c]Control
[d]Acidified and neutralized Control

there did not exist any consistent patterns of associative growth. Sc 5 and #7 produced subnormal amounts of acid; they were stimulated by all pasteurized cultures.

The Results of Prolonged Fermentation Within a pH Range to Detect Associative Growth

AM_2 and ML_8 were the most active in NDM with respect to the rate of acid production and the total acid produced for an 18 h period. Sl 1, #6, and #7 started with a slightly lower acid titration and paralleled AM_2 and ML_8. C_2, #8, and Sc 5 produced significantly less acid in 18 h. KH was relatively inactive. C_2 gradually decreased in acid production after 10 h. Sc 5 and #8 remained relatively inactive during the first 9 h and then started full acid production. Combinations of AM_2 with Sl 1, #6, and #7 were positioned between the two active cultures. Combinations of ML_8 with #8, KH, and C_2 paralleled one drop below the ML_8 culture. ML_8 + Sl 1, ML_8 + Sc 5, and ML_8 + #6 maintained an intermediate acid production rate. The patterns of combinations Sl 1 + #6, Sl 1 + #7, and #6 + #7 were identical to those of the single cultures. Combinations Sl 1 with C_2 and Sc 5 followed the pattern of Sl 1. Acid production of Sl 1 + KH, Sl 1 + #8, #7 +#8, #6 + #8, #6 + KH, and #7 + KH paralleled slightly below the most active culture.

Combinations of Sc 5 with #8, KH, and C_2 duplicated the acid production patterns of Sc 5. The combination #8 + C_2 paralleled slightly below C_2. The pattern established by #8 + KH paralled just below #8. Figures 1, 2, and 3 show typical titration curves of cultures neutralized with 20% NH_4OH over the 18 h. The activities of the cultures can be compared in Table 8.

No patterns of stimulation of the fast acid producers were observed, but slight inhibition of acid production by slow acid producers was noted in combinations. The rate of acid production and the total acid production of combinations was equal to or slightly less than the most active culture of the pair. No means by which dominance could be determined was observed.

No stimulation or marked inhibition of acid production by any cultures (Sl 1, Sc 3, Sc 4, Sc 5, #6, and #7) occurred when grown in NDM with a pasteurized culture (Sl 1, Sl 2, Sc 3, Sc 4, Sc 5, #6, #7, #8, KH, and R). Any difference greater than four drops NH_4Oh between the titration curve of the controls and the test cultures would have been considered possible associative growth patterns. When differences between the titration curves of the milk control and acidified-neutralized control occurred, the milk control consistently seemed 1-4 drops more active in all test deries. The exact reason was not investigated.

Sl 1 with pasteurized KH or #8 showed a decreased rate of

Figure 1. Prolonged fermentation in nonfat dry milk of combination Sl 1 + #8 to detect stimulation or inhibition. Fermentation maintained by neutralization with 20% NH_4OH

+---+ Sl 1, •---• #8, o---o Sl 1 + #8

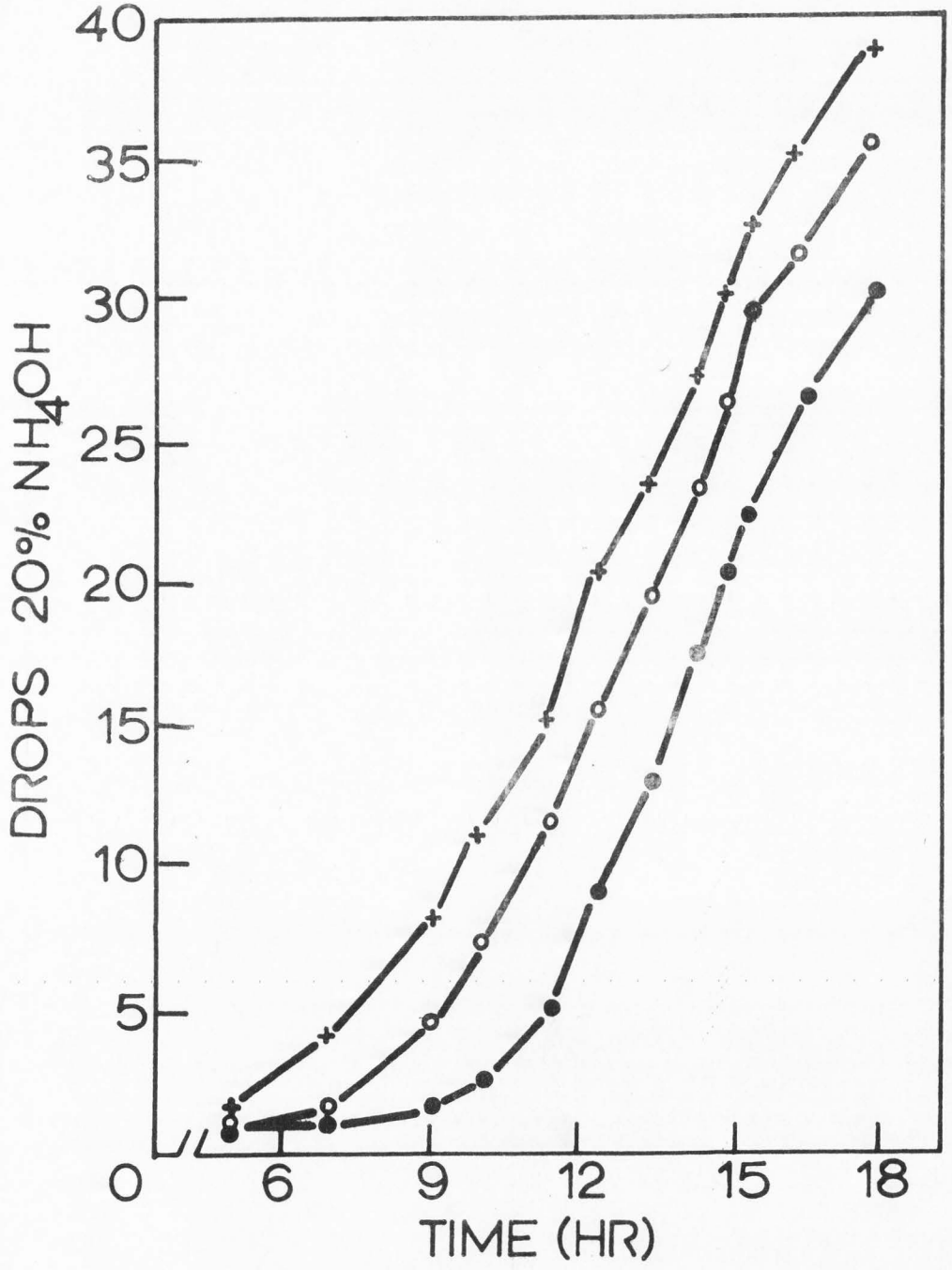

40
35
30
25
20
15
10
5
0
6
9
12
15
18
DROPS 20% NH_4OH
TIME (HR)

Figure 2. Prolonged fermentation in nonfat dry milk of combination Sl 1 + KH to detect stimulation or inhibition. Fermentation maintained by neutralization with 20% NH_4OH

+---+ Sl 1, ●---● KH, o---o Sl 1 + KH

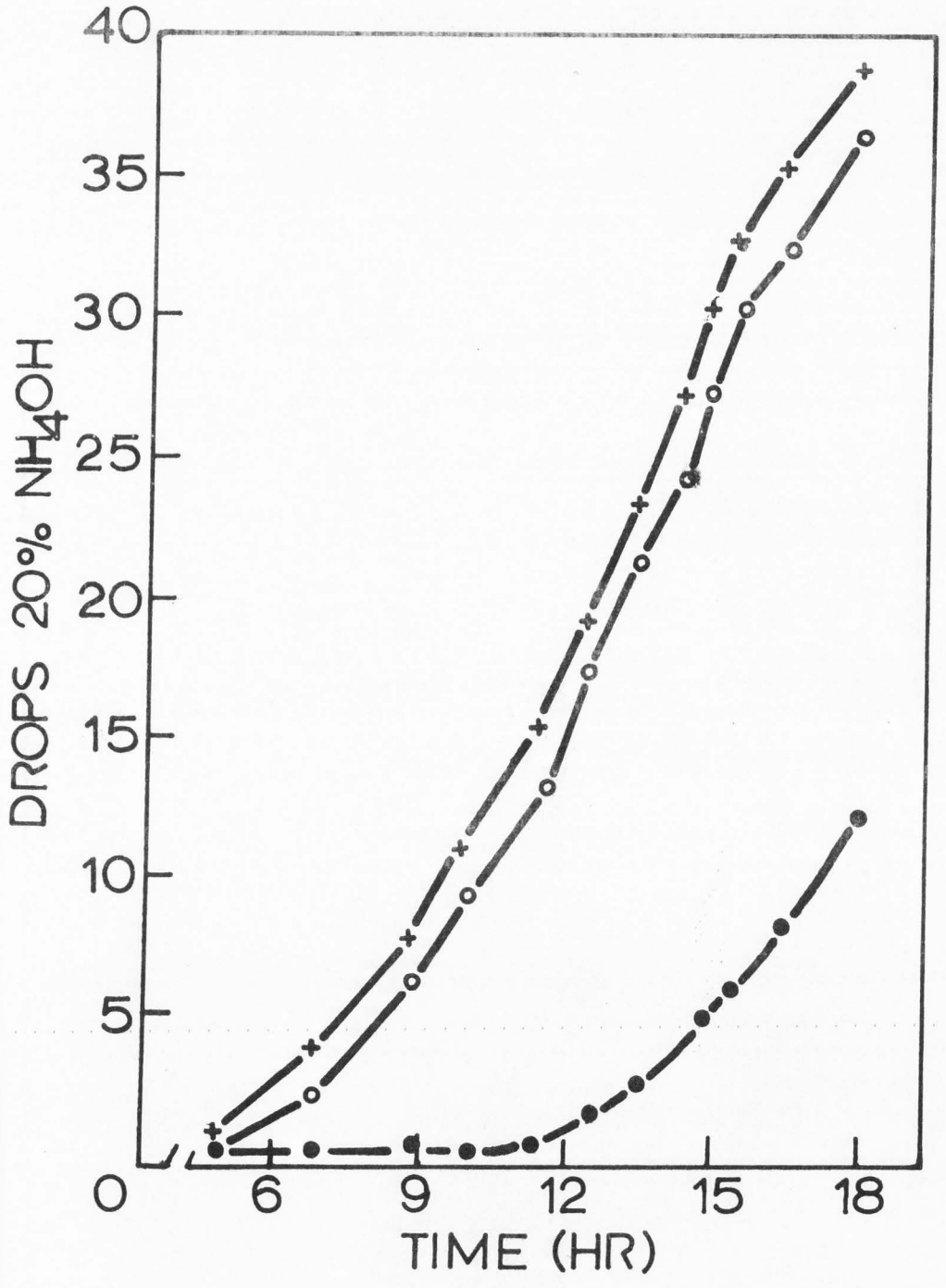

40
35
30
25
20
15
10
5
0
6
9
12
15
18
DROPS 20% NH4OH
TIME (HR)

Figure 3. Prolonged fermentation in nonfat dry milk of combination #6 + #7 to detect stimulation or inhibition. Fermentation maintained by neutralization with 20% NH_4OH

+---+ #6, ●---● #7, o---o #6 + #7

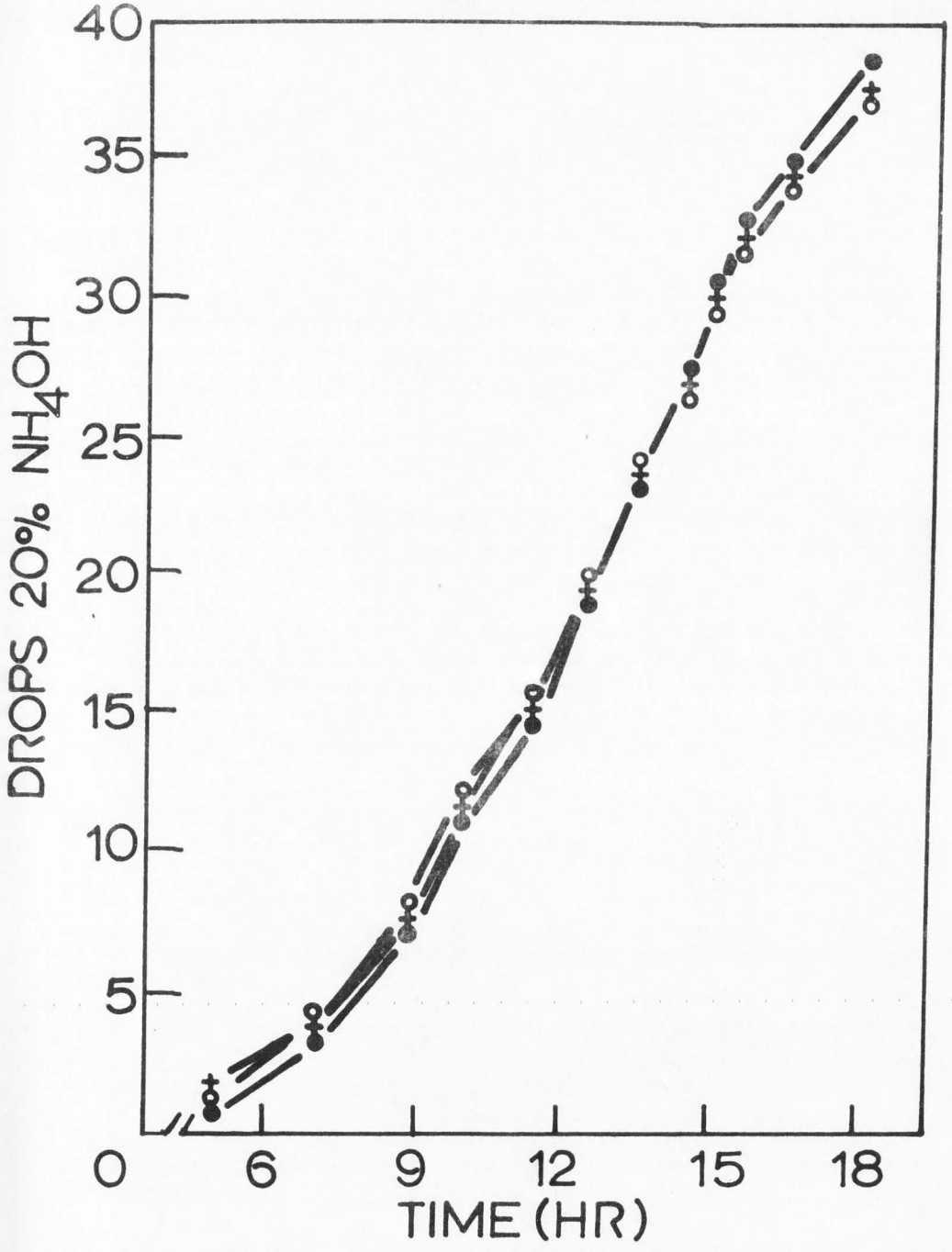

40
35
30
25
20
15
10
5
0
6
9
12
15
18
DROPS 20% NH_4OH
TIME (HR)

Table 8. A running total of drops of 20% NH_4OH used to maintain prolonged fermentation of single and combined cultures

	Time (hours)											
	5	7	9	10	11.5	12.5	13.5	14.5	15	15.5	16.5	18
Sl 1	1	4	8	12	16	20	24	28	30	33	36	40
Sc 5	1	1	3	6	10	14	18	22	25	28	31	34
#6	1	4	8	12	16	20	24	27	30	32	35	38
#7	1	4	8	12	16	20	24	28	31	33	35	39
#8	0	1	2	2	5	9	13	18	20	24	26	30
KH	0	0	0	0	1	2	3	4	6	7	9	13
AM_2	6	10	14	18	22	26	30	34	36	38	40	42
C_2	2	5	8	12	14	18	22	25	27	29	30	32
ML_8	3	7	11	15	19	23	27	30	33	35	37	39
Sl 1 + Sc 5	1	3	7	11	15	20	24	28	31	33	35	39
Sl 1 + #6	1	4	8	12	16	20	24	28	31	32	34	38
Sl 1 + #7	1	4	7	11	15	19	23	27	30	32	34	38
Sl 1 + #8	0	2	4	8	12	16	20	24	26	30	32	36
Sl 1 + KH	0	2	6	10	14	18	22	24	28	30	32	37
Sl 1 + AM_2	2	6	10	14	18	22	26	28	32	34	36	38
Sl 1 + C_2	0	4	8	12	16	20	24	26	30	32	34	38
Sl 1 + ML_8	2	6	10	14	18	22	26	29	31	33	35	38
Sc 5 + #6	1	2	6	10	14	18	22	25	28	30	32	35
Sc 5 + #7	1	3	6	10	14	18	22	26	29	32	34	37
Sc 5 + #8	1	2	3	6	10	14	18	22	25	28	30	34
Sc 5 + KH	1	2	2	5	9	13	17	21	24	26	28	31
Sc 5 + AM_2	1	6	10	14	18	22	26	28	30	32	32	34
Sc 5 + C_2	1	4	7	10	14	18	22	25	27	28	29	32
Sc 5 + ML_8	2	6	10	14	18	22	26	28	30	32	34	36
#6 + #7	1	4	8	12	16	22	24	27	30	32	34	37
#6 + #8	1	2	6	10	14	18	22	25	28	30	32	35
#6 + KH	1	2	5	8	12	16	20	24	27	29	31	34
#6 + AM_2	2	6	10	14	18	22	26	29	31	33	34	37
#6 + C_2	2	5	9	12	16	20	24	27	29	31	33	36
#6 + ML_8	2	6	10	13	17	22	26	29	31	33	35	38
#7 + #8	1	2	5	9	13	17	21	25	28	30	32	36
#7 + KH	0	2	5	8	12	16	20	24	27	30	32	36
#7 + AM_2	2	6	10	13	17	21	25	28	30	32	34	38
#7 + C_2	2	5	9	12	16	20	24	27	30	31	33	35
#7 + ML_8	2	6	10	14	18	22	26	29	31	33	35	38
#8 + KH	1	1	1	2	4	8	12	16	19	22	25	29
#8 + AM_2	2	6	10	13	17	21	25	28	30	32	33	36
#8 + C_2	1	3	6	9	12	16	19	22	24	26	27	30
#8 + ML_8	2	6	10	14	18	22	25	28	30	32	34	37
KH + AM_2	2	6	10	14	18	22	25	28	30	32	34	37
KH + C_2	1	3	6	9	12	15	18	21	24	26	27	30
KH + ML_8	2	6	10	14	18	22	25	28	30	32	33	36
AM_2 + C_2	2	6	10	14	18	22	25	29	31	33	36	38
AM_2 + ML_8	2	6	11	15	19	23	26	30	32	34	36	38
C_2 + ML_8	2	6	10	14	18	22	25	29	31	33	35	37

acid production as determined by neutralization with 20% NH_4OH (Figure 4). Generally, the combinations of Sl 1 with any of the pasteurized cultures decreased in acid production after 15 h. The total number of NH_4OH drops used was one to four drops less than the controls.

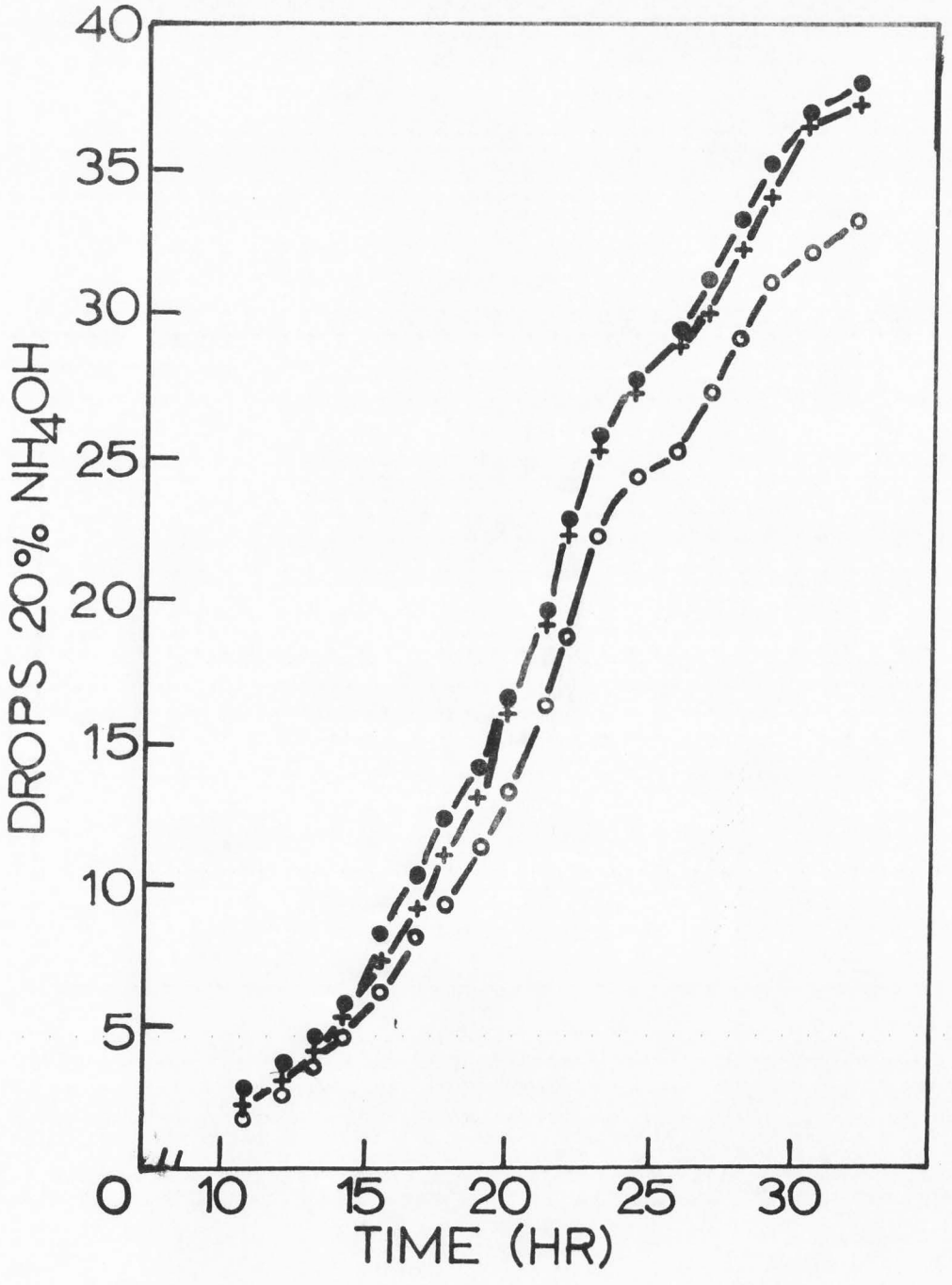
Sc 3 also exhibited the same pattern with pasteurized cultures Sc 3, Sc 4, #6, #7, and #8 (Figure 5). The reason for the abrupt stop in acid production by Sc 3 with pasteurized #6 was not determined, but was most likely the result of mass lysis by homologous phage as the inhibition occurred only in the one test.

Sc 4 and Sc 5 with the pasteurized cultures titrated somewhere between the two controls (Figure 6).

Cultures #6 and #7 with the pasteurized cultures produced acid equal to the neutralized controls. Figures 7-9 show that no significant stimulation or inhibition occurred.

When the test was performed in the whey-based medium, there was no significant stimulation or inhibition of cultures Sl 1, Sc 5, #6, and #7 by the pasteurized cultures Sl 1, Sl 2, Sc 3, Sc 4, Sc 5, #6, #7, #8, KH, R, or AM_2 (Figures 10 and 11). The results of Sc 3 and Sc 4 were discarded due to inactivity during the first 15 h. Some cultures produced more acid in the acidified, neutralized, and pasteurized control than in the pasteurized controls. The reason was not investigated.

Figure 4. A 30 h continuous neutralization curve of strain S1 1 with 20% of a 24 h neutralized, pasteurized strain of #8 or KH compared with the continuous neutralization curves of controls for evidence of stimulation or inhibition in nonfat dry milk. Neutralization curves of S1 1 with pasteurized strains #8 or KH were identical +---+ S1 1 in acidified-neutralized control, ●---● S1 1 in milk control, o---o S1 1 with pasteurized #8 or KH


40
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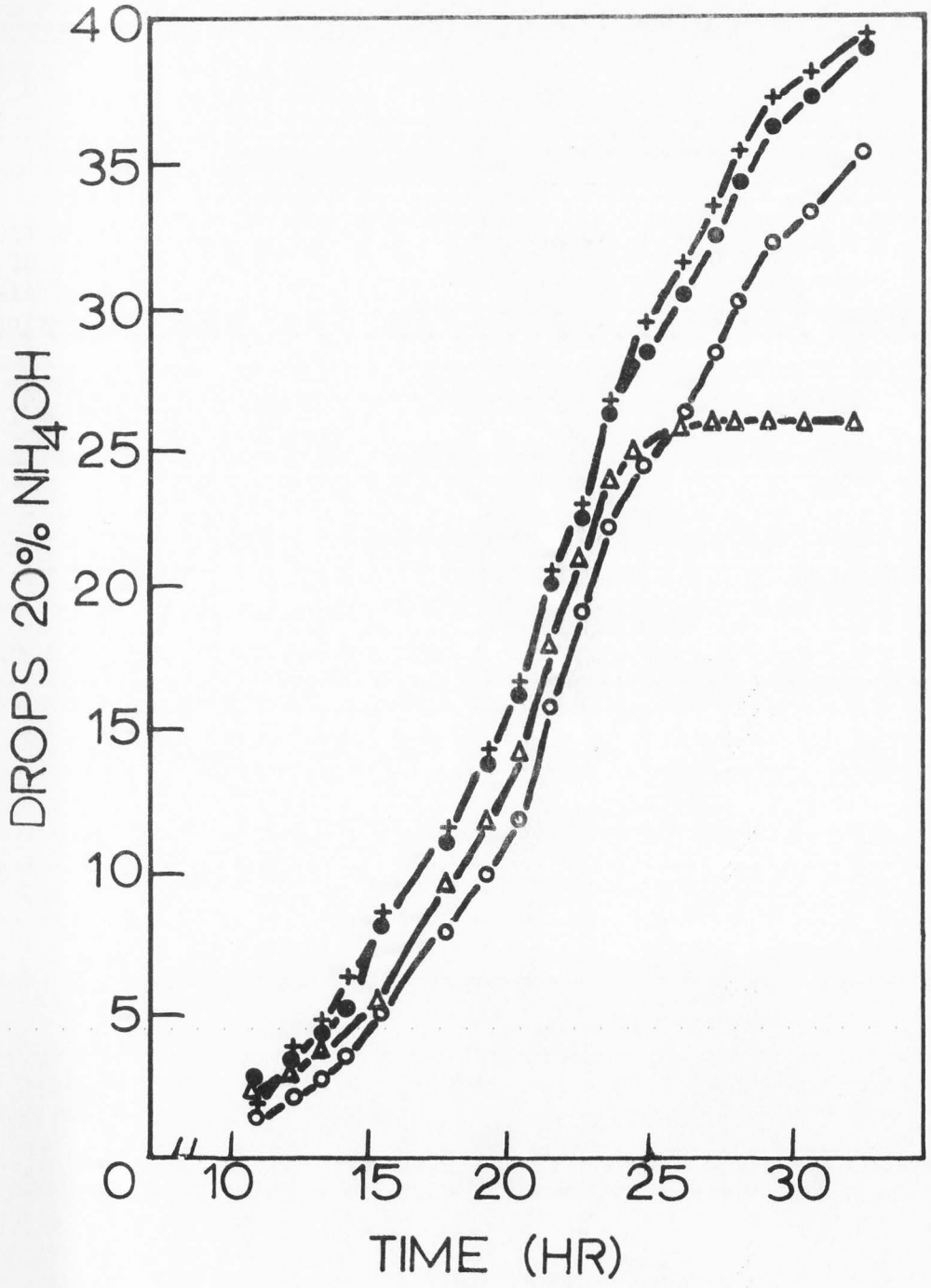
DROPS 20% NH_4OH
TIME (HR)

Figure 5. A 30 h continuous neutralization curve of strain Sc 3 with 20% of a 24 h neutralized, pasteurized strain of strains #6 or #7 compared with the continuous neutralization curves of controls for evidence of stimulation or inhibition in nonfat dry milk
●---● Sc 3 in milk control, +---+ Sc 3 in acidified-neutralized control, o---o Sc 3 with pasteurized #, ▲---▲ Sc 3 with pasteurized #8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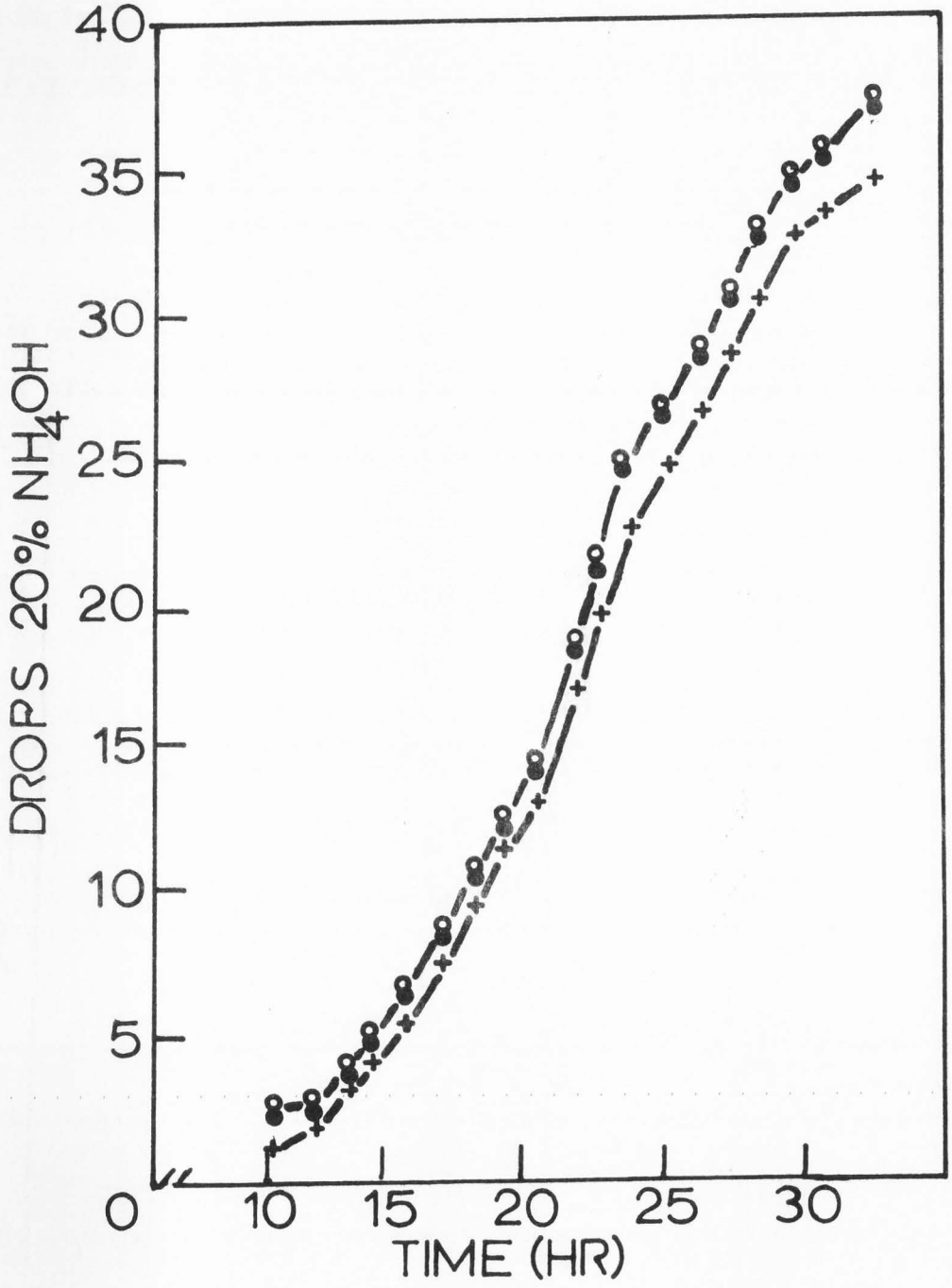
DROPS 20% NH_4OH
TIME (HR)

Figure 6. A 30 h continuous neutralization curve of strain Sc 4 with 20% of a 24 h neutralized, pasteurized strain of KH or R compared with neutralization curves of controls for evidence of stimulation or inhibition in nonfat dry milk
●---● Sc 4 in milk control, +---+ Sc 4 in acidified-neutralized control, o---o Sc 4 with pasteurized KH or R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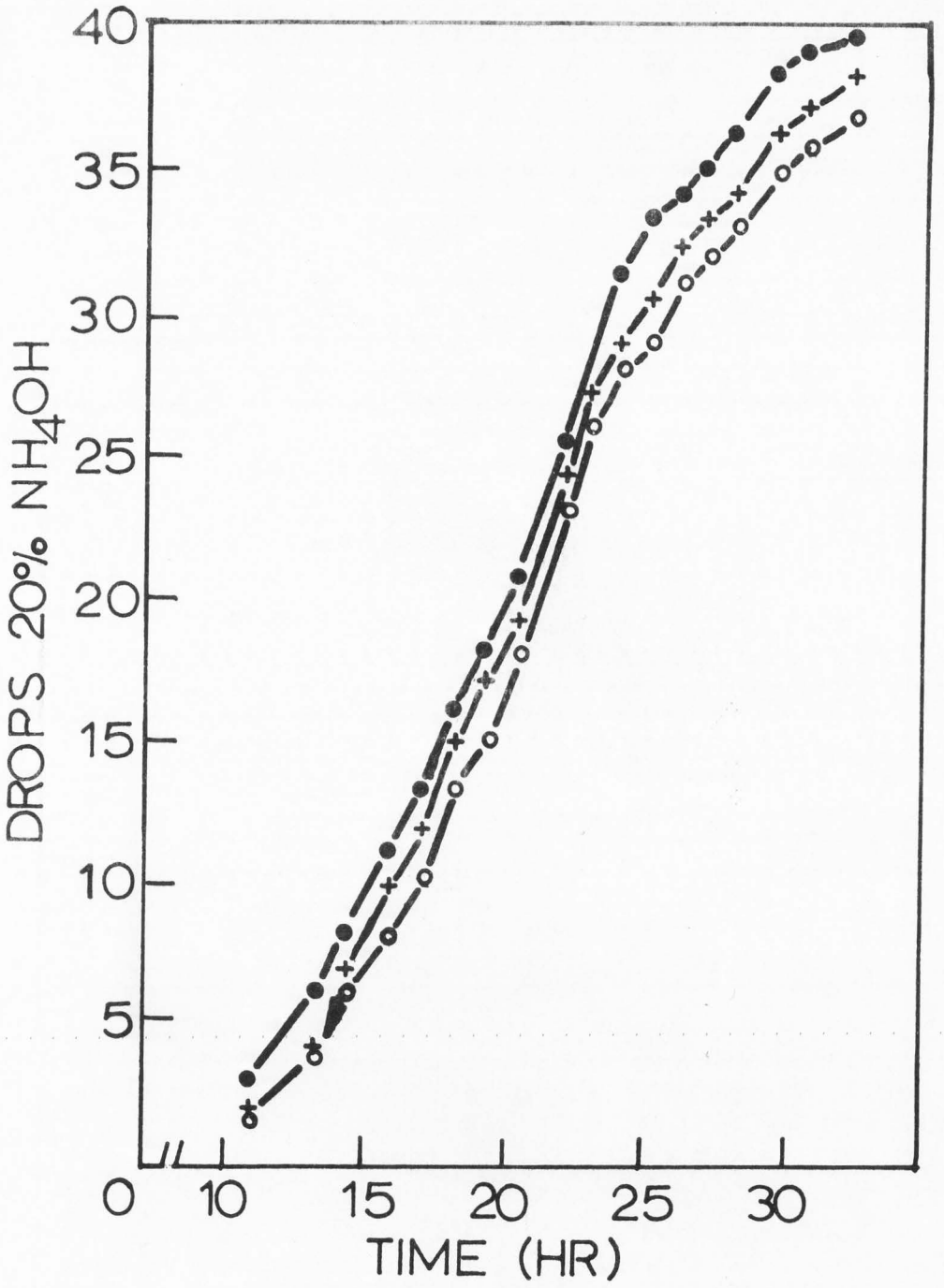
DROPS 20% NH_4OH
10
15
20
25
30
TIME (HR)

Figure 7. A 30 h continuous neutralization curve of strain #6 with 20% of a 24 h, neutralized, pasteurized strain S1 2 compared with the continuous neutralization curves of controls for evidence of stimulation or inhibition in nonfat dry milk
●---● #6 in milk control, +---+ #6 in acidified-neutralized control, o---o #6 with pasteurized S1 2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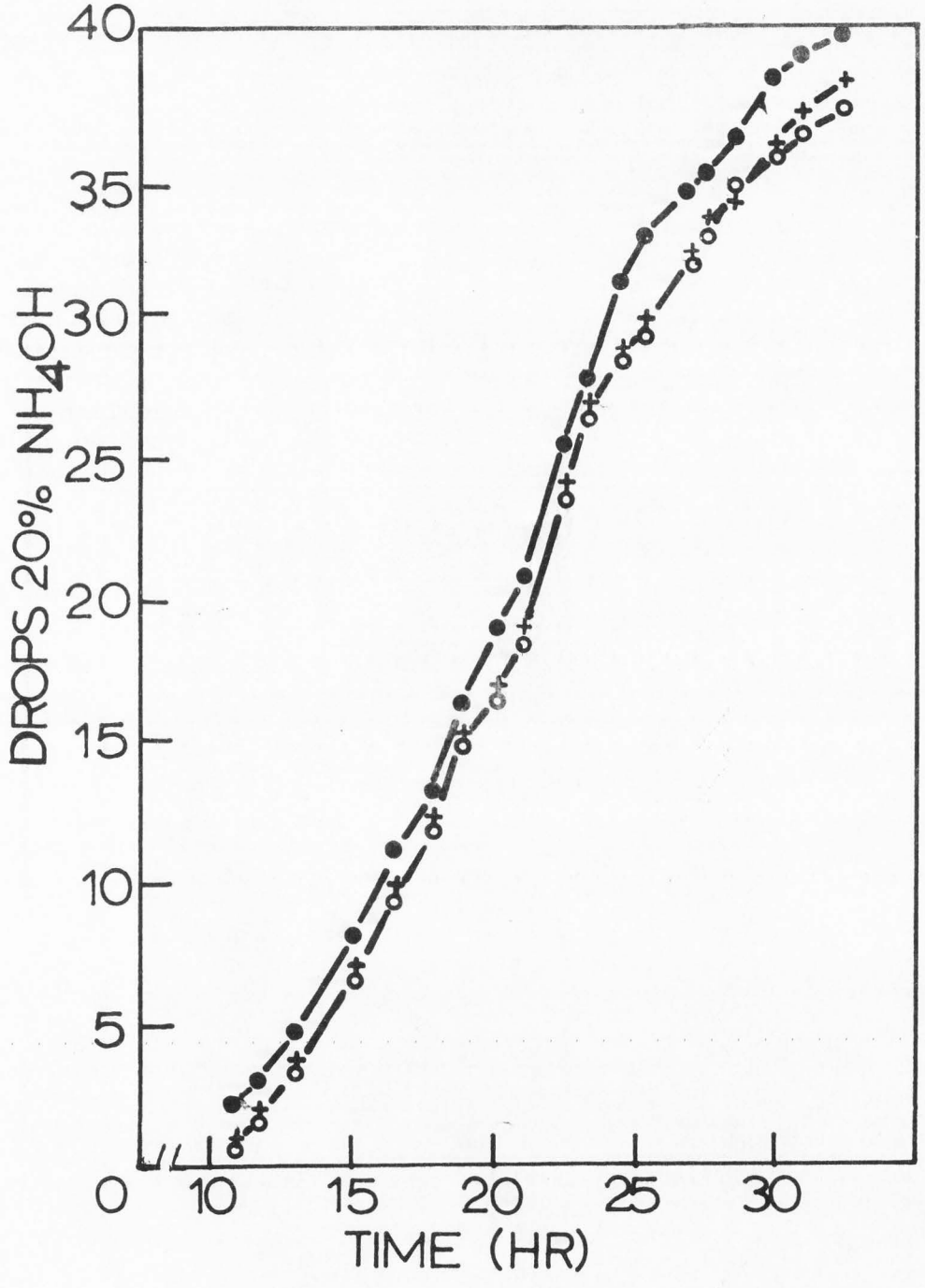
30
DROPS 20% NH_4OH
TIME (HR)

Figure 8. A 30 h continuous neutralization curve of strain #6 with 20% of a 24 h, neutralized, pasteurized strain #7 compared with the continuous neutralization curves of controls for evidence of stimulation or inhibition in nonfat dry milk
●---● #6 in milk control, +---+ #6 in acidified-neutralized control, o---o #6 with pasteurized #7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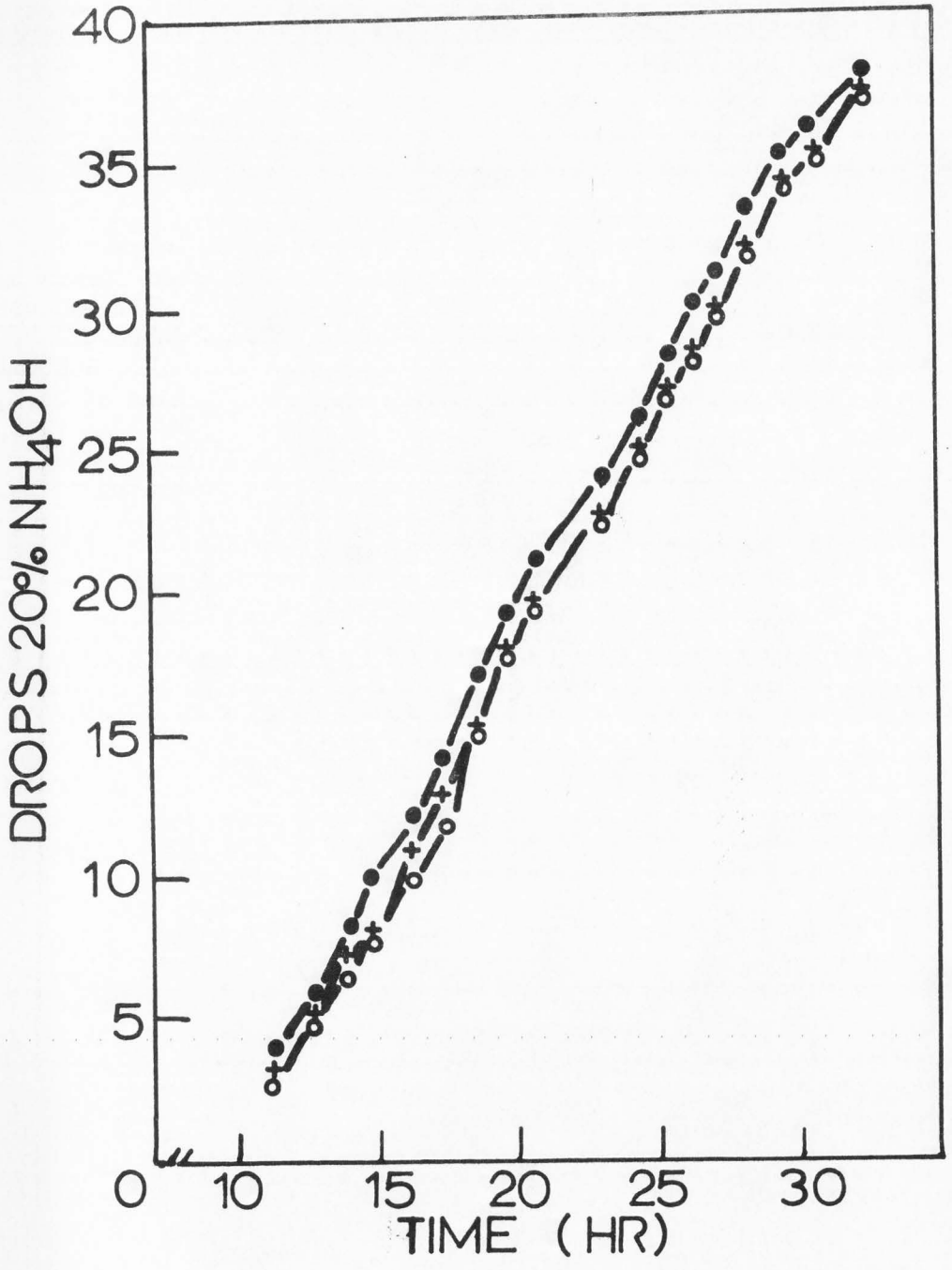
DROPS 20% NH_4OH
TIME (HR)

Figure 9. A 30 h continuous neutralization curve of strain #7 with 20% of a 24 h, neutralized, pasteurized strain of Sc 4, Sc 5, #6, or #7 compared with the continuous neutralization curves of controls for evidence of stimulation or inhibition in nonfat dry milk. Neutralization curves of #7 with pasteurized strains of Sc 4, Sc 5, #6, or #7 were identical
●---● #7 in milk control, +---+ #7 in acidified-neutralized control, o---o #7 with pasteurized Sc 4, Sc 5, #6, or #7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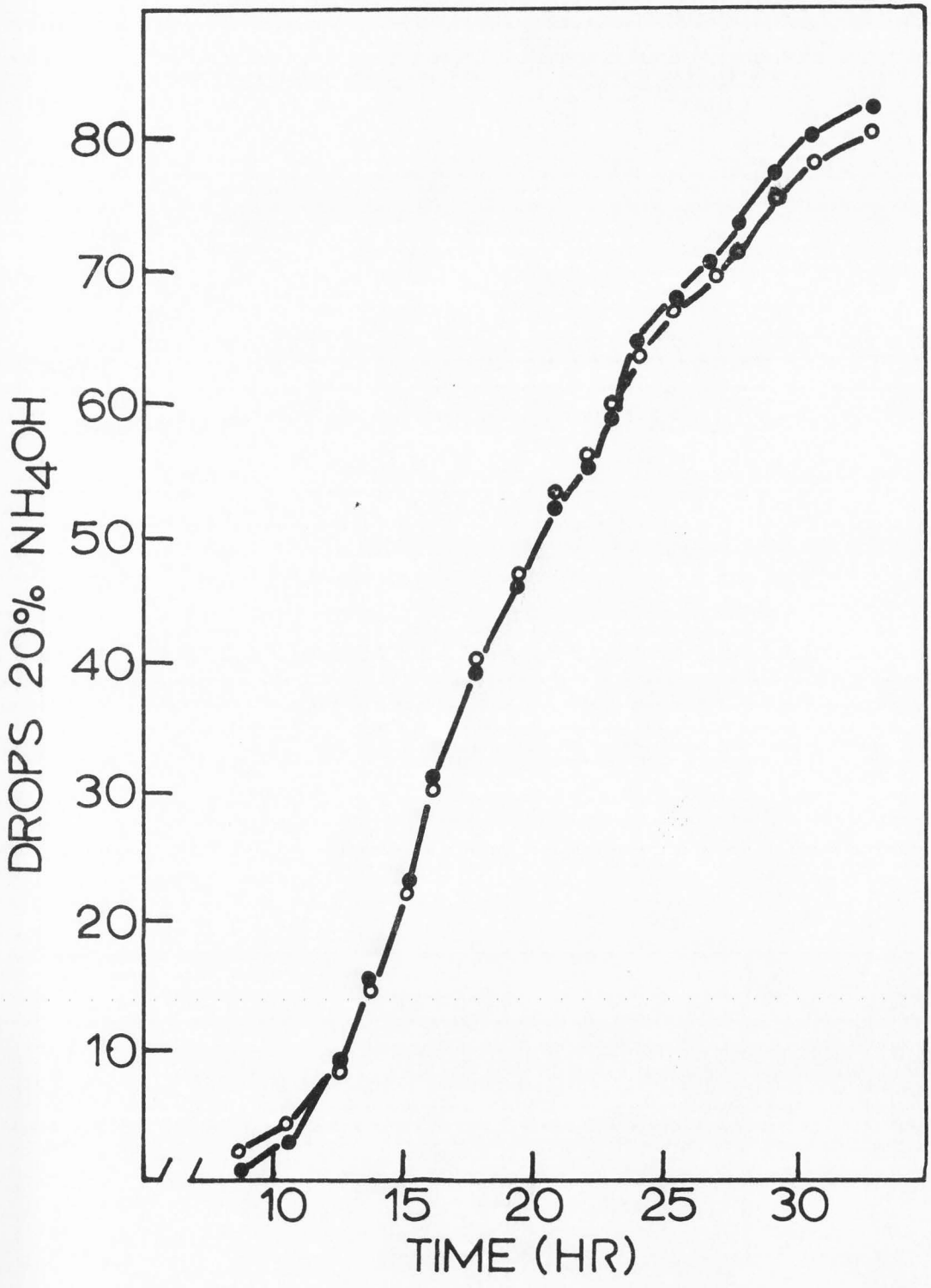
DROPS 20% NH4OH
TIME (HR)

Figure 10. A 30 h continuous neutralization curve of strain #6 with 20% of a 24 h, neutralized, pasteurized strain #7 compared with the continuous neutralization curves of controls for evidence of stimulation or inhibition in the whey-based medium
●——● controls, both neutralized and whey-based,
o---o #6 with pasteurized #7


DROPS 20% NH_4OH
80
70
60
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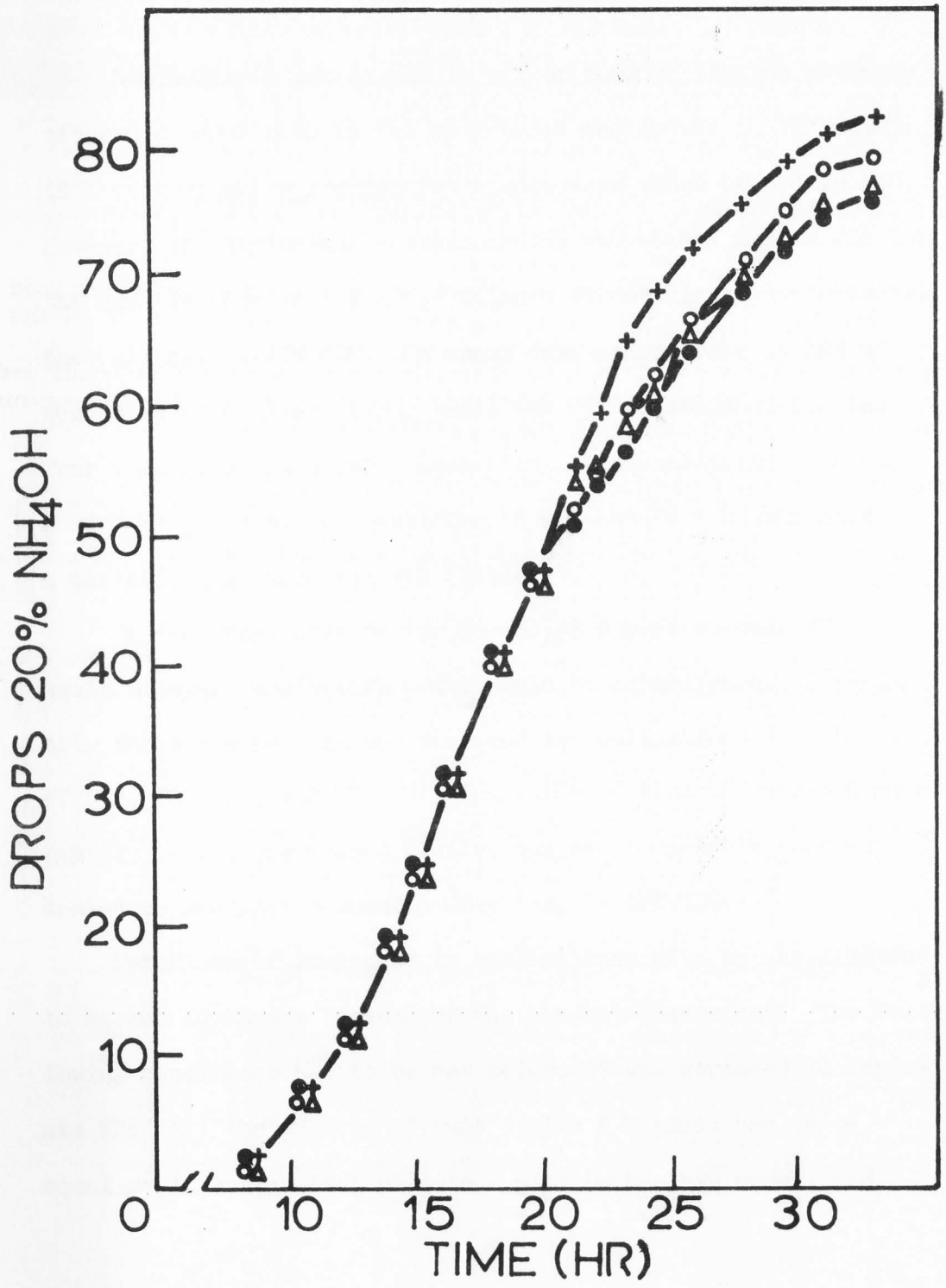
30
20
10
10
15
20
25
30
TIME (HR)

Figure 11. The effects of 20% of a 24 h neutralized, pasteurized strain #6 or #7 upon the acid production of #7 in the whey-based medium
●---● #7 in whey-based control, +---+ #7 in acidified-neutralized whey-based control, o---o #7 with pasteurized #6, ▲---▲ #7 with pasteurized #7


DROPS 20% NH4OH
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TIME (HR)

Dominance as Determined by the Phage Tracer Technique

Dominance by one strain in six or more of the ten combinations did occur both in the whey-based medium and in NDM within 48 h of continuous propagation within a pH range of 6.8 to 5.0. However, the domination pattern in the whey-based medium was not the same as that in 10% NDM. Culture #7 definitely dominated all the cultures in 10% NDM. An exact domination order in NDM as suggested by Collins (1955, 1961) was not established for the four remaining organisms. Each combination maintained its own dominance pattern. Combinations KH + R and #6 + R exhibited a variable dominance pattern (Table 9).

In the whey-based medium #6 and #8 dominated over #7. Again no exact domination order could be established. A variable dominance pattern was observed in combinations #6 + R, #7 + R, #8 + KH, and KH + R. Generally, dominance occurred more rapidly in the whey-based medium, however a variable sequence of dominance was more common in whey than in 10% NDM.

Detection of dominance in combinations #7 + KH was limited to marked dominance because of the nascent phenomenon. The following conditions had to be met before #7 was declared to dominate KH: (1) the pH drop of tube 3 with ϕ 7 added had to be equal or less than that of tube 2 with both phage races, and

Table 9. Dominance as determined by the phage tracer technique

Combinations		Test I		Test II		Test III		Test IV			
		Time (hours)									
		22.5	48	24	64	25	49.5	25	48	72	96
#6 + #7	M[a]	N[b]	#7	N	#7	N	#7	#7	#7	#7	#7
	W[c]	N	--	#7	#6	#6	#6	N	N	#6	#6
#6 + #8	M	N	#8	N	#8	N	N	N	N	N	N
	W	N	--	#8	N	N	N	N	N	N	N
#6 + KH	M	N	KH	N	KH	N	N	N	N	N	N
	W	#6	--	N	#6	#6	#6	#6	N	#6	#6
#6 + R	M	N	N	N	R	#6	#6	#6	R	N	R
	W	#6	--	N	N	#6	#6	R	R	N	R
#7 + #8	M	N	#7	#7	#7	#7	#7	#7	#7	#7	#7
	W	#7	--	#7	#8	N	#8	N	N	#8	#8
#7 + KH	M	#7	#7	#7	#7	#7	#7	#7	#7	#7	#7
	W	#7	--	#7	#7	#7	#7	#7	#7	#7	#7
#7 + R	M	#7	#7	#7	#7	#7	#7	#7	#7	#7	#7
	W	#7	--	#7	R	#7	#7	N	N	R	R
#8 + KH	M	N	KH	N	N	N	N	N	N	N	N
	W	N	--	KH	#8	#8	#8	#8	N	#8	#8
#8 + R	M	N	N	N	#8	N	#8	N	N	N	N
	W	N	--	N	N	#8	#8	N	--	--	--
KH + R	M	N	N	N	KH	N	N	R	N	N	N
	W	KH	--	KH	R	R	R	R	N	R	R

[a] Milk
[b] Neither dominated
[c] Whey-based Medium

(2) the pH decrease of tube 4 had to be equal or greater than that of the control. The following conditions had to exist before KH could be declared to dominate #7: (1) the pH decrease of tube 4 with ∅ KH added had to be equal or less than that of tube 2, and (2) the pH decrease of tube 3 had to be greater than that of tube 2.

DISCUSSION

Dominance does occur in the whey-based phage inhibitory medium as well as in NDM. The difference of dominance patterns supports previous conclusions that the media composition is a major factor in determining dominance. However, no exact domination order as suggested by Collins (1955, 1961) and by Lightbody and Meanwell (1955) was identified. Limiting the pH drop to a range of 6.8 to 5.2 did not significantly delay the occurance of dominance. The fact that the pendulum of strain dominance of supposedly compatable strains seemed to swing from one strain to the other strain supports the conclusion that no two active acid-producing lactic streptococci will grow in fixed proportion for long periods of time. The variable sequence of dominance also supports Collins' conclusion (1961) that competitive growth rather than some undetected antibiotic is responsible for dominance between non-antibiotic producing cultures. A comparison of the results of the associative growth experiments and the dominance tests of combination #6 + #7 tends to support the conclusion of Gilliland (1972) that a shift of the population may or may not affect the lactic acid production of a mixed cheese starter.

The phage tracer technique is an indirect method of determining dominance. It is limited to qualitative determinations.

It's usefulness is also limited by cross reactions caused by the lack of strain-specific phages and the nascent phenomenon. However, at present it is the most reliable method. The colony enumeration method of Reddy et al. (1969) and culture dialysis will give more direct evidence of dominance once problems are resolved.

If further studies are to be made, the method of Gilliland should be used. The growth of culture combinations should be controlled by a pH-stat with 20% NH_4OH at pH 6.3. Dominance could be determined at different time intervals. Dominance of combinations with pH control in the whey-based medium could be compared with dominance patterns of strain combinations with pH control in 10% NDM. These dominance patterns could then be compared with static conditions. Domination orders in mixed starters of three or more strains could be verified and compared with dominance patterns of two strain combinations by using the phage tracer method of Collins (1955) or another competent method.

The initial physiological state of the cultures studied seemed to determine whether stimulation or inhibition occurred when the cultures were grown in combinations. When the acid production of a culture was even slightly below its own norm, the normal activity was restored by substances present in a pasteurized or active culture. Frequently a series of pasteurized cultures restored activity equally well. When the slow

culture was grown with an active culture, it most often tended to retard the acid production of the active culture. The active culture is probably producing some essential metabolite which bypasses a metabolic hurdle of the slow culture. But as this metabolite is common to the metabolic pathways of both organisms a competative inhibition occurs.

CONCLUSIONS

1. Lactic acid in milk did not diffuse across a membrane in dialysis culture chambers.

2. The cultures used did not produce antibiotics.

3. Of the cultures studied, no consistent patterns of stimulation or inhibition were found when grown in combinations in the whey-based medium or NDM.

4. The acid production of a combination with one subnormal acid producer tended to be less than the single active culture.

5. The acid production patterns of combinations gave no indications of dominance.

6. Dominance by one strain in a combination of two lactic strains did occur in the whey-based phage inhibitory medium.

7. The domination patterns in the whey-based medium varied from those in NDM.

LITERATURE CITED

Anderson, A. W., and P. R. Elliker. 1953. The nutritional requirements of lactic streptococci isolated from starter cultures. I. Growth in a synthetic medium. J. Dairy Sci. 36:161-167.

Ausavanodom, Napasri. 1974. Phosphate requirements for a whey-based lactic bacteriophage resistant medium under pH control. MS thesis. Utah State University, Logan, Utah. p. 19-20.

Cheng, Chao Tung. 1970. Development of a whey-based lactic culture medium capable of bacteriophage inhibition. MS thesis. Utah State University, Logan, Utah. p. 16-17.

Collins, E. B. 1952 a. Action of bacteriophage on mixed strain starter cultures. I. Nature and characteristics of the "nascent phenomenon." J. Dairy Sci. 47:374-379.

Collins, E. B. 1952 b. Action of bacteriophage on mixed strain starter cultures. II. Relation to acid production of the proportion of resistant bacteria. J. Dairy Sci. 35:381-387.

Collins, E. B. 1955 a. Action of bacteriophage on mixed strain cultures. III. Strain dominance due to the action of bacteriophage and variations in the acid production of secondary growth bacteria. Appl. Microbiol. 3:137-140.

Collins, E. B. 1955 b. Action of bacteriophage on mixed strain cultures. IV. Domination among strains of lactic streptococci. Appl. Microbiol. 3:141-144.

Collins, E. B. 1961. Domination among strains of lactic streptococci with attention to antibiotic production. Appl. Microbiol. 9:200-205.

Czulak, J., and L. A. Hammond. 1954. Compatibility amongst strains of lactic streptococci. Aust. J. Dairy Technol. 9:15-18.

Dahiya, R. S., and M. L. Speck. 1962. Symbiosis among lactic streptococci. J. Dairy Sci. 45:607-612.

Dahiya, R. S., and M. L. Speck. 1963. Identification of stimulatory factor involved in symbiotic growth of Streptococcus lactis and Streptococcus cremoris. J. Bacteriol. 85:585-589.

Dahiya, R. S., and M. L. Speck. 1964. Growth of streptococcus starter cultures in milk fortified with nucleic acid derivatives. J. Dairy Sci. 47:374-379.

Elliker, P. R. 1951. The problem of bacteriophage in the dairy industry. J. Milk and Food Technol. 14:13-17.

Garvie, E. I., and L. A. Mabbitt. 1956. Acid production in milk by starter cultures--The effect of peptone and other stimulatory substances. J. Dairy Res. 23:305.

Gilliland, S. E. 1972. Strain balance of multiple strain lactic streptococcus concentrated cultures. J. Dairy Sci. 54:1129-1134.

Gilliland, S. E., and M. L. Speck. 1968. D-leucine as an autoinhibitor of lactic streptococci. J. Dairy Sci. 51:1573-1578.

Henning, D. R., W. E. Sandine, P. R. Elliker, and H. A. Hays. 1965. Studies with a bacteriophage inhibitory medium. I. Inhibition of phage and growth of single strain lactic streptococci and leuconostoc. J. Milk and Food Technol. 28:273.

Henning, D. R., E. R. Vedamuthu, W. E. Sandine, and P. R. Elliker. 1964. Citrate utilization and overgrowth by Streptococcus diacetilactis in mixed-strain starter cultures. J. Dairy Sci. 47:674.

Hirsch, A. 1952. The evolution of the lactic streptococci. J. Dairy Res. 19:290-293.

Hoyle, M., and A. A. Nichols. 1948. Inhibitory strains of lactic streptococci and their significance in the selection of cultures for starters. J. Dairy Res. 15:398-408.

Kothari, S. L., V. K. N. Nambudripad, and H. Laxminarayana. 1971. Studies on the associative growth of some streptococci. Milchwissenschaft. 26:415-418.

Kothari, S. L., and V. K. N. Nambudripad. 1973. Casein as a necessary factor in the production of stimulatory material for associative growth of lactic streptococci. Appl. Microbiol. 25:212-215.

Lightbody, L. G., and L. J. Meanwell. 1955. The growth of lactic acid streptococci in mixed starter cultures. J. Appl. Bacteriol. 18:53-65.

Mattick, A. T. R., and A. Hirsh. 1947. Further observations on an inhibitory substance (nisin) form lactic streptococci. Lancet. 153:5-8.

Moseley, W. K., and R. L. Winslow. 1959. A study of potential causes of culture failure in cheese factories. J. Dairy Sci. 42:906.

Nichols, A. A., and P. J. Ineson. 1947. Cheese starter "recovery" after attack by bacteriophage (strain dominance in multiple strain starters). J. Dairy Res. 15:99-111.

Oxford, A. E. 1944. Diplococcin, an anti-bacterial protein elaborated by certain milk streptococci. Biochem. J. 38:178-182.

Reddy, M. S., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold. 1969. Differential agar medium for separating Streptococcus lactis and Streptococcus cremoris. Appl. Microbiol. 18:755:759.

Reddy, M. S., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold. 1971. Associative growth relationships in two strain mixtures of Streptococcus lactis and Streptococcus cremoris. J. Milk and Food Technol. 34:236-240.

Reddy, M. S., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold. 1972. Agar medium for differential enumeration of lactic streptococci. Appl. Microbiol. 24:947-952.

Schultz, J. S. and P. Gerhardt. 1969. Dialysis culture of microorganisms: Design, theory, and results. Bacteriol. Rev. 33:1-47.

Vedamuthu, E. R., B. A. Sauser, D. R. Henning, W. E. Sandine, P. R. Elliker, and E. A. Day. 1966. Competitive growth of Streptococcus diacetilactis in mixed strain lactic cultures and cheese. Proc. 17th Inter. Dairy Congr. D(2):611-618.

Vedamuthu, E. R., and G. W. Reinbold. 1967. Starter cultures for cheddar cheese. J. Milk and Food Technol. 30:247-252.

Whitehead, H. R., and G. J. E. Hunter. 1941. Starter cultures for cheese manufacture--further attempts to eliminate failures due to bacteriophage. J. Dairy Res. 12:63-65.

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